University of Alberta

Isolation and Characterization of Transcripts Related to Salt Stress Responses in *Pinus banksiana L.* (Jack Pine) and *Picea mariana* (P. Mill) (Black Spruce)

by

Rose Marie Lacson - Guardamano

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

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ABSTRACT

Two conifer species, jack pine (*Pinus banksiana Lamb.*) and black spruce (*Picea mariana* [Mill.] B.S.P.), were screened for salt tolerance using sodium chloride (NaCl) solutions at 0.00 mM, 85.51 mM, 119.72 mM, 171.03 mM, and 256.54 mM concentrations. The plant materials used included two hundred two jack pine and three black spruce cell lines generated by somatic embryogenesis, 600 seeds of jack pine and 600 seeds of black spruce germinated in selective media in vitro, and 74 four-and-a-half-month-old seedlings of jack pine germinated in the soil. Expressed transcripts in response to the treatments were isolated via the Differential Display Reverse Transcription-Polymerase Chain Reaction (DD RT-PCR) technique coupled with Polyacrylamide Gel Electrophoresis (PAGE) and autoradiography. A total of 301 differential expressed fragments representing transcripts of up-regulated and down-regulated genes were identified. Of these, 64 fragments were isolated, cloned and sequenced. Forty-seven fragments represented up-regulated genes and eight represented down-regulated genes responding to salt stress. Sequence-specific primers were designed to confirm and quantify their expressions by RT-PCR. Fourteen fragments showed significant similarities to entries in GenBank and 23 fragments showed significant sequence similarities to entries in the Swiss Institute of Bioinformatics (SIB). Thirty-three fragments did not have any significant sequence similarities with any of the entries in the GenBank and the SIB. These might be novel genes that are specific to jack pine and black spruce. Some of the fragments were similar to the Japanese black pine chloroplast hypothetical protein, *Pinus pinaster* partial mRNA for putative metallothionein-like protein, *Pinus eliotti PEC* 30 mRNA, Picea *abies* mRNA for membrane intrinsic protein, different types of *Pinus taeda* xylem wood mRNA and mRNA sequence of *Pinus Taeda* triplEx pollen cone. Comparison of these fragments to published sequences of salt tolerant genes did not show significant similarities. The results of this study suggest that the same salt stress response mechanisms working in other plants are also employed by jack pine and black spruce, however, a different set of genes may be responsible for the same functions in these trees. This study provided further evidence of the possibility of success in generating jack pine and black spruce

clones through somatic embryogenesis using mature seeds. It also provided additional evidence of intraspecific variability in the responses to salt stress in jack pine and black spruce.

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ABBREVIATIONS

APS	ammonium persulfate
ATISC	Alberta Tree Improvement and Seed Centre
Acc#	accession number
ATPases	adenosine triphosphatases
BAP	6-benzylaminopurine
BLAST	Basic Local Alignment Search Tool
BS	black spruce
Ca	calcium
Ca^{2+}	calcium ion
Cl^-	chloride ion
cDNA	complementary deoxyribonucleic Acid
CTAB	hexadecyltrimethyl ammonium
dATP	diadenosine triphosphate
DEPC	diethylpyrocarbonate
DBI	double bond index
DD	Differential Display
DD RT-PCR	Differential Display Reverse Transcription-Polymerase Chain Reaction
DNA	deoxyribonucleic acid
dNTP	dinitrogen triphosphate
DRE	dehydration response element
EDTA	disodium ethylenediaminetetraacetic acid
ET	embryogenic tissue
fm	femtomole
GH	GenHunter
g	gram

GSP	gene specific primer
HCl	Hydrochloric acid
H^+-pase	inorganic pyrrophosphatases
JP	jack pine
JPZE	jack pine zygotic embryos
K	potassium
K^+	potassium ion
Kb	kilobase
LiCl	lithium chloride
LM	Litvay Medium
Na^+	sodium ion
NAA	1-napthalene acetic acid
NaCl	sodium Chloride
NET	non-embryogenic tissue
ml	milliliter
mg	milligram
mRNA	messenger ribonucleic acid
MMLV	Moloney Murine Leukemia Virus Transcriptase
MS	Murashige and Skoog
NaOAC	sodium acetate
NO_3^-	nitrate
P	phosphorus
PCR	Polymerase Chain Reaction
pm	picomole
PVP	polyvinylpyrrolidone
QTL	Quantitative Trait Locus
RNA	ribonucleic acid
RPM	revolutions per minute

RT	reverse transcription
T1/T2/T3	Treatment 1/2/3
S	sulfur
SAS	Statistical Analysis Software version 8.01 TS level 01MO
SDS	sodium dodecyl sulfate
U	Unit
µg	microgram
µL	microliter
V	volt
ZE	zygotic embryo

CHAPTER I

INTRODUCTION

Increased soil salinity is an ecological concern mainly because of the resulting loss of productivity of agricultural lands (Kawasaki *et al.* 2001, Kasuga 1999, Steppuhn & Wall 1997) and its impact on water quality (Quist & Williams 1999). Much of the arable land in the world is becoming increasingly salinized through irrigation practices and limited water availability (Winicov & Blastula 1997). This is further aggravated by global climate change, which may lead to water deficits in many parts of the world. Also contributory are some commercial fertilizers that interact with chlorinated water to produce sodium chloride (NaCl). Salinity in the soil affects about 7% of the earth's land surface and about 5% of the cultivated land (Flower *et al.* 1997). Most importantly, about 20% of the irrigated lands have suffered from secondary salinisation and 50% of irrigation schemes are affected by salts (Flower *et al.* 1997).

The impact of elevated salinity on forests has not received the attention it has in agriculture. It is nevertheless, a widespread and costly problem (Allen *et al.* 1994). Excess salinity has affected afforestation projects in India (Mathur & Sharma 1984) and roadside trees in the northern United States and Canada (Dochinger & Townsend 1979). Salinity is also an issue with the reclamation of land near oil, gas, or salt mining facilities (Rincon 1980, Auchmoody & Walters 1988). In northeastern Alberta, oil sands mining tails have substantially increased salinity of the soil. Currently, the re-vegetation of saline environments with native tree species such as jack pine (*Pinus banksiana Lamb.)* and black spruce (*Picea mariana* [*Mill.*] B.S.P.) is challenging because of their high mortality (Fung *et al.* 1998).

In a study of the susceptibility of 13 pine species *to* NaCl spray, jack pine was among the least tolerant species while *P. thunbergii* and *P. ponderosa* were the most tolerant species (Townsend &

Kwolek 1987). However, forest trees have demonstrated high levels of variation within species for salt tolerance (Sykes 1992, Zekri & Parsons 1992, Redfield & Zwiazek 2002, Epstein *et al.* 1998, Downton 1984, Mohammed & Rumbaugh 1989), including jack pine (Yeatman 1975). Van der Moezel *et al.* (1989) found the highest intraspecific variability in salt tolerance occurring in the species that were on the average, the least tolerant. This might suggest that a sizeable increase in salt tolerance is possible by identifying and selecting salt-tolerant trees in species traditionally thought to exhibit a low level of tolerance to salt, like jack pine and black spruce.

Screening for salt tolerance is often performed by growing seedlings in high salinity solutions (Renault *et al.* 1999, Nicknam & McComb 2000). This is expensive due to the need for screening of a large number of seedlings in order to find a few salt-tolerant trees. A plausible alternative is to select for marker gene associated QTL that confer salt tolerance in tree species. Marker-assisted selection (MAS) for salt tolerance has been deployed in agriculture, in rice (*Oryza sativa*) (Flower et *al.* 2000) and tomatoes (*Lycopersicum esculentum*) (Monforte *et al.* 1996). To the best of my knowledge, there is no report on MAS for salt tolerance in forest trees. A comparison of molecular markers between trees that are tolerant and susceptible to salt stress might identify those markers associated QTL that increase or decrease salt tolerance. These molecular markers will be valuable for selecting native trees to become the source of salt tolerant seeds for propagation. Planting of seedlings derived from these select trees in salt-affected areas would be cost-effective and environmentally responsible.

Analysis of the mRNA from the tissue of a plant exposed to environmental stress, such as high salinity, could lead to the identification of genes encoding proteins that impart salt tolerance thereby setting the foundation for improvement. Determinants of plant stress tolerance have been identified in organisms such as yeast (*Saccharomyces cerevisiae*), Arabidopsis (*Arabidopsis thaliana*) and peas (Pisum sativum L.) (Hasegawa et al. 2000). The mechanisms and the determinants for salt tolerance have been elucidated only in the past few decades of the 20th century by using mostly

unicellular models such as bacteria, yeast and algae (Hasegawa et *al.* 2000). Although similarities in the mechanisms and determinants of salt tolerance could exist among trees and these model organisms, it is probable that there are mechanisms and determinants of salt tolerance that are unique to forest trees.

There is a dearth of information on the genetic basis of salinity stress responses in trees. Most of the studies on salt stress were conducted on agricultural crops such as garlic (*Allium sativum*) (Siddiqui *et al.* 1996), wheat (*Triticum aestivum*) (Nemoto *et al.* 1999), rice (*Oryza sativa*)(Gong *et al.* 1999), sorghum (*Sorghum vulgare*) (Koyro 1997), celery (*Apium graveolens*) (Pardossi *et al.* 1999), barley (*Hordeum vulgare L.*)(Mano & Kayzuyoshi 1997), alfalfa (*Medicago sativa*) (Petrusa & Winicov 1997), cotton (*Gossypium hirsutum*) (Chen Cui 2000), and tomatoes (Foolad & Chen 1998). Compared with agricultural crops, relatively little progress has been made with either conventional or biotechnological methods in understanding salinity stress responses in trees, although studies are underway now in several countries (Allen 1994, Niknam & McComb 2000).

The aim of my study is to lay the groundwork for understanding the genetic basis of salt stress response in jack pine and black spruce. My study objective was to identify, isolate and characterize transcripts related to salt stress responses in jack pine and black spruce. My study was divided into two parts. Part 1 dealt mainly with screening for salt stress responses to obtain suitable materials for DD-RT-PCR. Part 2 focused on the isolation and identification of these transcripts resulting from differentially expressed genes. I believe my study is the first important step toward understanding the genetic mechanisms of salt tolerance in jack pine and black spruce at the molecular level.

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Chapter II

Review of Literature

1. Impact of Salt Stress on Plants

Salt stress commonly caused by high Na^+ and Cl^- concentration in the soil (Hasegawa *et al.* 2000), affects plants structurally and physiologically, which in turn decreases productivity (Steppuhn & Wall 1997). Plants vary in their responses to salt stress depending on their tolerance to it.. In buffalo grass [*Buchloe dactyloides (Nutt.) Eagelm.*], NaCl reduced the amount of plasma membrane phospholipids mainly serine and inositol of the anionic phospholipids, in salt sensitive clones compared to salt-tolerant clones (Lin & Wu 1996). NaCl also increased the number of double bonds per mole, and reduced the plasma membrane H^+-ATPase activity and the total plasma membrane protein concentration (Lin & Wu 1996). Salinity decreased root branching in all subspecies of *Salsola kali* in Europe (Rilke & Reimann 1996) and reduced the aboveground and root biomass in *Dalbergia sissoo Roxb* (Singh *et al.* 1996). In barley treated with salts, the root tip meristematic cells showed disruption in internal structure, vesiculated plasmalemma, accumulation of lipid droplets, vacuolation of cytoplasm, and swelling to a complete damage of mitochondria (El-Banna & Attia 1999).

One commonly observed effect of elevated NaCl is the suppression of plant growth (Rilke & Reimann 1996, Fung *et al.* 1998, Hasegawa *et al.* 2000) resulting from the inhibition of cell division. This was reported for shoot growth in wheat cultivars (Steppuhn & Wall 1997), celery (Pardossi *et al.* 1999), and in two Sudanese guava (*Psidium guajava L.*) cultivars (Ali-Dinar *et al.* 1999). NaCl also reduced germination in asparagus (*Asparagus officianales*), table beet (*Beta vulgaris*) and sea aster (*A. laurentianus*) (Uno *et al.* 1996). At high concentration, NaCl completely inhibited germination, reduced seed maturation and growth of seedlings in sea

aster (Uno *et al.* 1996). Salinity stress reduced leaf expansion (Munns & Termaat 1986) and at high concentration inhibited nitrogenase activity, diminished root nodule number and dry matter accumulation (Abd-Alla *et al.* 1998).

One known effect of salinity stress on plants is the reduction of substrate water potential. This restricts nutrient uptake causing ionic imbalance, nutrient deficiency, and toxicity (Greenway and Munns 1980). NaCl stress causes attenuated acquisition of the nutrients phosphorus (P), potassium (K) (Wu *et al.* 1996), calcium (Ca), nitrate (Pardossi *et al.* 1999) and sulfur (S) (Koyro 1997). NaCl stress also reduces the concentration of K in roots and leaf petioles. Nutrient reduction is less pronounced in the young compared to the mature leaves (Pardossi *et al.* 1999). Calcium translocation was more inhibited by sodium in the salt sensitive wheat Modoc than in the tolerant *Triticum aestivum c.v. Kharchia* (Davenport *et al.* 1997). According to Hasegawa *et al.* (2000)), a hypersaline environment, most commonly mediated by high NaCl, results in perturbation of the ionic steady state not only for Na^+ and Cl^-, but also for K^+ and Ca^{2+} (Niu *et al.* 1995). External Na^+ negatively impacts intracellular K^+ influx, attenuating acquisition of this essential nutrient by the plant cells (Niu *et al.* 1995).

Salinity has been shown to affect photosynthesis in *Populus euphratica* Olive and the hybrids [*P. tallasica,* Kom *X (P. euphratica x Salix alba L.)*] (Ma *et al.* 1997). High salt treatment reduced photosynthesis not because of damage to the photosynthetic apparatus but more likely due to inhibition of the dark reaction (Ma *et al.* 1997). In the guava tree, saline condition reduced leaf chlorophyll levels (Ali-Dinar *et al.* 1999). Salinity also induced clear changes in the ultra-structure of chloroplasts. The most notable change was a disoriented lamellar system, with the grana and intergrana lamellae becoming swollen. The effect was greater in salt sensitive than the salt-tolerant genotypes of barley (El-Banna & Attia 1999). In the present study, some of these structural or physiological impacts of salinity stress were observed in jack pine and black spruce. They were used as indicators for the selection of tolerant individuals or cell lines for DD-RT-PCR.

2. Plant Responses to Salt Stress: the Mechanisms

Depending on their sensitivity to the presence of salt in their growth substrate, plants can be categorized as glycophytes or halophytes, the former being sensitive to salt and the latter being salt loving. The glycophytes responses to saline stress and the mechanisms by which they survive saline stress are generally different from the halophytes. With the advent of biotechnology in the early 21st century knowledge on the mechanisms of salt tolerance in plants is accumulating. Halophytes that are exposed to salinity stress appear to maintain active homeostatic mechanisms at all times. Conversely in glycophytes, the protection mechanisms needs time in order to activate such that when exposed suddenly to full complement of salt the plants go into shock and die. Gradually exposing the plants over a long period of time would allow them to develop some tolerance. One notable difference between glycophytes and halophytes is that the former responds to salt stress by an arrest of growth, while the latter continues to grow or gradually slow down their growth rates under extreme conditions (Michalowski *et al.* 1989).

Whether glycophytes or halophytes, plants develop ways to reduce the impact of salt stress. This can be either avoidance or tolerance. Avoidance refers to the ability to keep salt ions away from parts of the plants where they would be harmful. Avoidance occurs when a plant prevents or reduces damage without reaching equilibrium with the stress. Plants that are tolerant to salt stress achieve equilibrium with the stress, and as a result do not experience serious injury (Redfield & Zwiazek 2002). Avoidance occurs by passive exclusion of ions because of membrane permeability, active extrusion via ion pumps or dilution through the development of succulent tissue. This is accommodated by compartmentalization in the vacuoles and in the corresponding osmo-regulation in the cytoplasm (Greenway & Munns 1980, Levitt 1972).

Other known mechanisms related to plant avoidance or tolerance of salt stress include stomatal closure, which mitigates ion flux to shoots, (Hasegawa *et al.* 2000); accumulation of large quantities of ions in mature and old leaves (Pardossi *et al.* 1999), which may eventually dehisce; entry of ions into the xylem stream, the symplastic ion transport through the epidermal and cortical cells and the endodermis (Hasegawa *et al.* 2000), thus sparing actively growing and metabolizing cells from toxicity. This is a kind of structural adaptation whereby meristematic cells, which are not directly connected to the vasculature, are less exposed to ions delivered through the transpiration stream. Their small vacuolar spaces are not conducive to ion storage, and are therefore predominantly occupied by osmolytes (Hasegawa *et al.* 2000).

Another mechanism by which plants deals with salt stress is production or accumulation of compatible solutes such as proline (Liu & Zhu 1997, Serrano1996, Petrusa & Winicov 1997, Hoberg 1998), mannitol or fructans (Serrano 1996) that serve as osmolytes for increasing the osmotic potential or as competitive substrates for salts. These organic solutes may accumulate in the cytosol, the lumen, or the stroma of organelles (Hasegawa *et al.* 2000). Some plants exhibit tolerance through ionic selectivity. Calcium is implicated for its function in conjunction with salt stress signaling pathways that mediate salt tolerance (Pardo *et al.* 1998, Liu & Zhu, 1998) and in ion selectivity, aiding in the accumulation of K^+ and the exclusion of Na^+ (Epstein 1998, Hasegawa *et al.* 2000, Liu & Zhu 1997).

Halophytes ultimately survive and grow in saline environments because of osmotic adjustment through intracellular compartmentalization that partitions toxic ions away from the cytoplasm (Waisel 1991) through energy-dependent transport into the vacuole (Hasegawa *et al.* 2000). The study of Flower *et al.* (1997) suggested that glycophytes are incapable of maintaining ionic gradients necessary for vacuolar compartmentalization of the ions. However, Binzel *et al.* (1988) demonstrated that the membrane properties required in achieving and maintaining such gradients did exist in glycophytes, but the rate at which they

can establish the steep gradients was much slower than in halophytes. Sorghum cultivar 'Sweet Sioux', a glycophyte, sequesters NaCl into the epidermal vacuoles and cortex cells (2 cm behind the root tip), requiring a high level of energy consumption (Koyro 1997). Halophytes exclude Na^+ and Cl^- through glands and bladders. Halophytes have more responsive Na^+ partitioning and more effective capacity to coordinate this partitioning with processes controlling growth and ion flux across the plasma membrane, in both the cellular and the organismal context (Hasegawa *et al.* 2000).

The mechanisms of tolerance played by vacuoles in plants include regulation of cytoplasmic pH; sequestration of toxic ions and xenobiotics; regulation of cell turgor; storage of amino acids, sugars and carbon dioxide in the form of malate; and possibly as a source of elevating cytoplasmic calcium (Barkla & Pantoja 1996). These are driven by two primary active transport mechanisms in the tonoplast. Energy is required in response to salt stress, as these primary transport activities require a high-energy metabolite for their function.

Jack pine and black spruce used in this study are glycophytes. Like other forest trees, the mechanisms by which some individuals within the species showed tolerance to elevated salinity are not yet well understood. Knowledge of mechanisms of salt stress response derived from other plants may be used in the understanding mechanisms involving forest trees. But, because of differences in life history traits between trees and annual plants there may be mechanisms unique to trees that will be revealed through studies of trees themselves.

3. Determinants of Salt Tolerance

Salt tolerance was reported to be a multigenic trait (Foolad 1997, Hoberg 1998, Flower *et al.*1997, Winnicov 1998, Zhong & Dvorak 1995). This suggests that the apparent tolerance to salt stress was mainly the result of several genes working towards the mitigation of the

effects of elevated salinity. Studies of barley (Mano & Kayzuyoshi 1997), tomatoes (Foolad 1996) and cotton (Chen Cui et *al.* 2000) have shown these genes to have additive effects.

Hasegawa *et al.* (2000) categorized the genetic determinants of salt tolerance as either effector molecules (metabolites, proteins, or components of biochemical pathways) that lead to adaptation or as regulatory molecules (signal transduction pathway components) that control the expression and activity of the effectors. These effector determinants may function in ionic homeostasis as transmembrane transport proteins including H^+ translocating ATPases and pyrophosphatases involved in secondary active transport and electrophoretic flux across the plasma membrane and tonoplast (Na^+ and Cl^- vacuolar compartmentalization), Ca^{+2} ATPases (Calcium $^{2+}$ transport systems, secondary active transporters and channels) (Hasegawa *et al.* 2000).

Vacuolar antiporter (Na^+/H^+ antiporter) (Fukuda *et al.* 1998, Apse *et al.* 1999) transports Na^+ from cytoplasm to vacuoles. Influx of sodium into the tonoplast vesicle was accelerated by a pH gradient. This was caused by adenosine 5'-triphosphatase (H^+-ATPase) (Zhang *et al.* 1999) and the proton-translocating inorganic pyrrophosphatase (H^+-ppase) (Gaxiola *et al.* 1999). Tonoplast antiporters are constitutive in halophytes, whereas they must be activated by NaCl in salt-tolerant glycophytes, and they may be absent from salt-sensitive glycophytes (Glenn *et al.* 1999).

Advancements in molecular techniques paved the way to identify salt tolerant genes and to investigate the determinants of salt tolerance at the gene level. Some genes like CBF 1(CRT/DRE) are regulatory genes that work not only in response to salt stress but also to other abiotic stresses such as freezing and drought (*Kasuga et al. 1999*). Other genes code for enzymes such as methyl transferase. This enzyme is found in some fungi, marine algae and halophytic plants. It functions in the control and regulation of the internal concentration of chloride ions in halophytic plant cells (Ni & Hager 1999).

Several effector determinants were studied in *Arabidopsis thaliana*. These include a salt overly sensitive (SOS) 1 gene, salt tolerance genes encoding a putative Na^+/H^+ antiporter (Huazhong *et al.* 2000); SOS 3 (Liu & Zhu 1997), a single gene locus which encodes a signal transduction intermediate that modulates high-and low-affinity K^+ uptake (Hasegawa *et al.* 2000); and ACA 4 gene that encodes a vacuolar membrane calcium pump (Geisler *et al.* 2000). Calcium plays very important role in the alleviation of toxicity due to salt stress. It is primarily implicated in transport of ions across membranes. Under salt stress, calcium ions are displaced by Na^+ and this calcium deficiency results to greater sensitivity mainly due to inadequate compartmentalization and excessive ion accumulation in the cytoplasm (Kinraide 1999). Yeast calcineurin also known as PP2B, a regulator of ion homeostasis in yeast cells (Mendoza *et al.* 1996) functions in conjunction with the salt stress signaling pathway in plants to mediate salt tolerance (Pardo *et al.* 1998).

Another effector determinant is a high affinity potassium sodium symporter HKT 1 found in wheat root (Rubio & Gassmann 1995). In *Arabidopsis thaliana,* a K^+ channel AKT 1 gene expressed in a yeast strain defective for K^+ uptake at low K^+ concentration restores K^+ transport in this strain, and it increased tolerance to NaCl or Lithium Chloride (LiCl), whatever the external K^+ concentration used (Ros *et al.* 1999). The NHA 1 gene in *Saccharomyces cerevisiae* encodes a protein mediating Na^+ and K^+ efflux through the plasma membrane required for alkali cation tolerance at acidic pH (Banuelos *et al.* 1998). The PMR 2-gene cluster encodes functionally distinct isoforms of a putative Na^+ pump in the yeast plasma membrane involved in Na^+ tolerance. (Wieland *et al.* 1995).

Examples of regulatory determinants are HAL 1 (Bordas *et al.* 1997) and TPS 1 from yeast. They are involved in stress tolerance in transgenic plants and improved salt tolerance under *in vitro* culture conditions (Serrano *et al.* 1999). Hal 3p is a yeast halo tolerance determinant (De Nadal & *Clotet* 1998). Hal 4 and Hal 5 are protein kinases that modulate TrK1 and TrK2 potassium transporters (Mulet *et al.* 1999). Another regulatory determinant is

DRE, a dehydration response element. It is a cis-acting promoter element that has a role in regulating gene expression in response to stresses (drought, salt loading, and freezing) (Kasuga *et al.* 1999). A comprehensive list of these effectors and regulator determinants from yeast and plants was tabulated in a review by Hasegawa *et al.* (2000). The sequences of the fragments isolated in this study were compared with the sequences of some of these determinants such as CNA, DBF 2, HAL1, TPS 1, SOS 3, DREB/CBF, SAL 1, MEKK1, CDPK1, GSK1, and CBL1. The purpose was to determine whether the fragments from jack pine and black spruce are related to these genes.

Allen *et al.* (1994) stated that the knowledge of physiological mechanisms responsible for difference in salt tolerance has not been used effectively for screening. To date, progress on this area is still slow, and not much has been done to improve salt tolerance especially in forest tree species. To ultimately understand plant responses to salinity stress, it is imperative to isolate and identify the genes implicated in these responses and then to assess their functions.

4. Differential Display RT-PCR as a Method of Isolating and Characterizing Transcripts Related to Salt Stress Responses.

Drs. Arthur Pardee and Peng Liang invented DD RT-PCR in 1992 at Harvard Medical School to allow rapid, accurate and sensitive detection of altered gene expression (GenHunter Corporation (1996-2002). It is a powerful new tool for identifying and cloning differentially expressed genes (GenHunter Corporation (1996-2002). The concept of differential display is to use a limited number of short arbitrary primers in combination with anchored oligo-dT primers to systematically amplify and visualize most of the mRNA in a cell (GenHunter Corporation 1996-2002). This technology works by systematic amplification of terminal positions of messenger ribonucleic acid (mRNA) and resolution of those fragments on a deoxyribonucleic acid (DNA) sequencer (GenHunter Corporation 2001-2002). In a

comparison with 5 other techniques commonly used since 1992, namely subtractive hybridization, representational differential analysis (RDA), RNA-arbitrarily primed PCR (RAP-PCR), DNA microarray and serial analysis of gene expression (SAGE), differential display quickly became the method of choice for cloning (GenHunter Corporation 1996-2002). Differential display can examine several different RNA samples simultaneously. It measures semi-quantitative differences in gene expression rather than the complete absence or presence of different mRNA species. It is a robust technique, isolation of mRNA is not required and comprehensive analyses consumes only about 15 micrograms of total RNA from each cell population. DD can also detect novel genes (GenHunter Corporation 2001-2002). DD RT-PCR does however; require identical genotypes for treatment so that the difference in expression is the reflection of treatment rather than the genetic background. In this study, clones derived from somatic embryogenesis were used to meet this requirement. For seedlings germinated in vitro, the tissues were pooled into 5-7 seedlings per sample. For seedlings germinated in the soil, tissue samples were taken before and after treatment from the same individual plant. Another consideration in using DD RT-PCR is that the products are short (less than a thousand base pairs). These short fragments, can however be aligned and mapped with known genes to determine their identity. Differential display has been used to isolate up-regulated (Zhang 1996, Nemoto *et al.* 1999) and down-regulated (Li & Chen 2001) genes induced by salt stress in plants. GenHunter Corporation reports 1545 papers using this technique. In this study DD RT-PCR was the main technique used in the isolation of fragments associated with up-regulated and down-regulated genes in NaCl stress.

DD-RT-PCR utilized extracted and purified total RNA. Integrity of total RNA can be checked by running in formamide gel. Purity, absorbency, ratio should be within acceptable values, and RNA should be intact. Using Moloney Murine Leukemia Virus (MMLV) reverse transcriptase and oligo-dT primer, the RNA is reverse transcribed into complementary DNA (cDNA). The fragments are amplified through Polymerase Chain Reaction (PCR) using an oligo (dT) primer, a random primer, and a lower concentration of dinucleotide triphosphate

(dNTP) (25 μM). To visualize the fragments on sequencing gels, diadenosine triphosphate (dATP) can be labeled radioactively, fluorescently, or with infrared dyes, or primers used can be labeled. The bands of interest are excised, and extracted from the gel. The excised fragments are amplified using the same set of conditions as the first PCR step but without labeling.

5. Selection of Stable Variants for DD RT-PCR

As stated earlier, several studies recognize the existence of variations among individual plants, provenances or cultivars, within populations, and within families. Differences in the ability to exclude Na^+ or Cl^- has been observed in Citrus (Maas 1993), eucalyptus species (Thomson 1988), *P. radiata* (Cromer at al 1982) and numerous annual crop species (Greenway & Munns 1980). Jack pine and black spruce are generally salt-sensitive species, which exhibit variability within populations. It is therefore possible that from these salt sensitive species one can isolate a few salt tolerant individuals. It is however important that these variants be genetically stable. That is, they should possess the desired trait, which is heritable, and the tolerance they exhibited should not just be due to epigenetic changes but should be expressed constitutively throughout the plant's life span (Winicov 1994). Careful selection of stable variants is a crucial step in the isolation of genes for salt tolerance and was carefully considered in the screening for salt tolerant individuals.

In this study, the embryogenic tissues were in culture for not more than three months before they were subjected to salt treatment. The study of Winicov (1994), selecting for cellular salt tolerance with alfalfa, suggests culturing prior to selection for not more than three months because prolonged subculturing increases the occurrence of epigenetic changes. Winicov (1994) also suggests the use of callus in selection for cellular salt tolerance because it is likely to introduce fewer genetic rearrangements than selection in suspension culture.

6. Somatic Embryogenesis and Isolation of Genes for Salt Tolerance

One complicating factor to the isolation of genes for salt tolerance is the multigenicity of the trait (Foolad 1999, Hurkman 1992) and the complex pattern of inheritance in trees (Cooper & Gorton 1952, Furr and Ream 1969, Sykes 1992). Despite the complex pattern exhibited by species investigated to date, it is apparent that genes with a major effect (Allen 1994) on salt tolerance can be found (Abel 1969, Newman & Artcliff 1984). The salt tolerance is also known to develop with age (Ashraf & Khanum 1997, Ashraf & O'Leary 1997) and in glycophytes, tolerance is usually expressed strongly with gradual exposure, rather than immediate exposure to a high level of salt (Steppuhn & Wall 1997).

Currently there are many techniques in molecular biology that can be used to isolate and characterize genes. One of the most commonly used is Differential Display Reverse Transcription Polymerase Chain Reaction (DD RT-PCR). The use of cloned tissues in DD RT-PCR facilitates the isolation of transcripts representing differentially expressed genes that are associated to salt treatment rather than to other factors such as genetic differences of the source tissues. Tissue culture has been used for many years to screen stable variants possessing certain desirable traits. It was believed that careful choice and manipulations of tissues in culture could make DD RT-PCR effective for use in jack pine and black spruce in this study.

Somatic embryogenesis (SE) was the tissue culture method of choice for generating clones for DD RT-PCR mainly because of its reported success in jack pine and black spruce using immature zygotic embryos as explants (Natural Resources Canada). SE includes 4 phases: initiation and proliferation of embryogenic tissue, maturation of somatic embryos, germination of somatic embryos, greenhouse culture and fields planting. This method generates unlimited numbers of genetically identical intact plants by the formation of embryos

similar to zygotic embryos. The tissues can be cryogenically preserved and later allowed to resume development; therefore SE can provide an almost perpetual source of tissue for further study. It has been reported by the Canadian Forest Service than in pine it is much more difficult to obtain somatic embryos and mass propagate a large numbers of clonal lines.

In vitro selection for salt tolerance utilizing plant cell culture has recently received increased attention (Winicov 1994) as a viable alternative to traditional plant breeding. In this study it is considered as an adjunct technique providing the source tissue to isolate genetic markers. These markers can be useful in isolating the genes responsible for the desired character salt tolerance, which in turn can be used to assess individual trees ideal for plant breeding, reforestation, and phytoremediation. Pre germination tissues (embryogenic tissues with surrounding calli) and seedlings at germination in vitro were screened for tolerance to salt stress. This early selection and screening in vitro have been done in earlier studies by Foolad (1999) in his study " Comparing salt tolerance during seed germination and vegetative growth in tomato by QTL mapping", and by Grieve (1999) in the study entitled " Screening eucalyptus clones for salt tolerance".

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CHAPTER III

SCREENING FOR PLANT RESPONSES TO SALT STRESS

1. Introduction

Salt tolerance is defined in this study as the ability of juvenile jack pine (JP) and black spruce (BS) to survive the effects of high salinity. This study identifies, isolates, and characterizes molecular markers for salt tolerance in JP and BS through DD RT-PCR. This technique works best when genetically identical source tissues are subjected to salt treatment. Such genetic material eliminates the background genetic noise so that the observed response is due to the salt treatment. Genetic identity was achieved by using clonal lines. Somatic embryogenesis (SE) provided sufficient amounts of cloned tissues for the different treatments required for this study. For JP and BS seedlings germinated and treated *in vitro*, five to seven seedlings from same treatment, family, and species were pooled together to make a sample for "control" and "treated" respectively. For the four-and-half-months-old seedlings each plant was considered individually. Sample for "control" was taken from pretreated tissue and sample for "treatment" was from post-treated tissue of the same individual.

Since it has been reported that salt tolerance develops with age (Shannon *et al.* 1984), and that different genes work at different stages of development (Mano & Kayzuyoshi 1997, Foolad 1996), seedlings germinated and treated *in vitro* and four-and-a-half-months-old seedlings planted in soil were included in the screening.

Salinity stress can be due to several different types of salts. In this study NaCl was used because it is the most commonly found salt in soil (Hasegawa *et al.* 2000), and it has been used in the majority of studies on salt stress. Salt concentration of 0 mM to 256.54 mM NaCl

were used in this study. The concentration used was based on those use successfully in many salt stress studies. The baseline concentration used in the selective media was 85.51 mM. NaCl concentrations of 0.00 mM–256.54 mM were sufficient to trigger an observable difference in response, discriminating tolerant from the non-tolerant individuals. The rational behind the use of varying concentrations of NaCl was to determine the range of salt concentration that can be tolerated by JP and BS. Some of the previous studies used only a single concentration for selection, and it was possible that JP and BS could tolerate a much higher concentration than that tested previously tested. Also, an original objective was to correlate gene expression with varying degree of salt stress.

2. Materials and Methods

2.1 Plant materials

The Alberta Tree Improvement and Seed Center (ATISC) provided all the seed that were used in this study. Approximately 500 seed from each of the six families of jack pine and six families of black spruce were received on September 21, 1999. The seed from the jack pine families designated ATISC Acc# 4194, 4195, 4196, 4199, 4208, and 4209 were collected in 1998 within the range of 57.10.00 to 59.19.13 latitude and 111.02.00 to111.32.26 longitude, within an elevation range of 300-480 meters. The seed from black spruce families designated ATISC Acc.# 4160, 4162, 4163, 4164, 4282, and 4292 were collected within the range of 54.04.00 and 57.10.20 latitude and 111.24.20 to 116.37.00 longitude. The seed from the first five families of black spruce were collected from the seed orchard at the ATISC. The seed from first four families of black spruce were collected in 1997, while the seed from the fifth and sixth families were collected in 1998 and1999 respectively. The seed of all the JP and BS families had 87.5% to 99.5% germination rates.

2.2 Somatic embryogenesis of jack pine and black spruce

2.21 Surface sterilization of mature jack pine (JP) and black spruce (BS) seeds

The procedures used in this part of the study were adopted from "A Laboratory Guide to Somatic Embryogenesis in Spruce and Larch" (Lelu et. al. 1993) provided by Dr. Y.S. Park, and Dr. Krystyna Klimaszewska of Natural Resources Canada. According to Dr. Klimaszewska, immature zygotic embryos of JP and BS were the source of explants originally found to have embryogenic capacity. They have never succeeded in inducing somatic embryogenesis from mature JP seed. This study started in October 1999 and only mature JP seeds were available as a source of explants. The protocol originally designed for immature BS seed was tried with mature JP embryos.

Approximately 60-80 seeds from each family of JP and BS were placed in separate tea strainers. They were immersed in a 1000-ml beaker with 800 ml of 20% sodium hypochlorite (NaOCL) bleach (Javex) solution to which 5 drops of the surfactant Tween 20 was added. The beaker was covered with aluminum foil and was placed on an electromagnetic stirrer. The solution was stirred at maximum speed for seven minutes, to ensure the flow of solution around the seeds. The seeds in the tea strainers were rinsed twice for 10 minutes each by electro magnetically stirring them in a covered sterile beaker containing 800 ml of sterile distilled water. The seed were then transferred to 15 X 25-ml sterile disposable petri dishes (Sigma-Aldrich Canada Ltd., Oakville, Ontario) containing 25 ml of double distilled deionized water and soaked overnight. Soaking the seeds overnight facilitated the dissection of the fragile zygotic embryos.

2.22 Excision of the explant and induction of the embryogenic tissues

A stereomicroscope was surface-disinfected by spraying it with 70% ethanol. It was placed in a laminar flow unit previously sterilized by spraying it with 95% ethanol. The spatula, scalpels, and forceps were autoclaved and further sterilized by flaming (dipped in 100% ethanol and burned three times) just before use.

The seeds were scooped out of the water using a spatula and dissected. Using a scalpel, the partially split seed coat was carefully sliced open. While holding one end of the seed opposite the radicle with a forceps, the seed coat was pulled away from the megagametophyte. The megagametophyte was carefully sliced open to expose the fragile embryo. The embryo was picked with the forceps and transferred to a modified gelled half-strength Litvay medium supplemented with 2, 4-D (2.2mg/ml), BAP (1.1 mg/ml)) and kinetin (1.1mg/ml). Ten seed were plated in a 100 X 15-mm petri dish containing 25 ml of the medium. A total of fifty seed from each family for each species were plated in five petri dishes. The petri dishes were labeled JP 1A 1B 1C 1D 1E for Jack pine family 1, plate a, b, c, d, and e respectively. Plates of zygotic embryos from family 2 were labeled JP 2A, 2B, 2C, 2D and 2E. Black spruce zygotic embryos from family 1 were labeled BS 1A, 1B, 1C, 1D, and 1E. The rest of the plates from the other families in both species were labeled in the same way. The plates were sealed with parafilm and incubated in a dark growth chamber set at 25°C for 2 weeks. The first lot of seed was plated October 14, 1998 to October 29, 1998. Second lot of seed was plated November 1 to November 26, 1998. In the first lot, 50 zygotic embryos for each of the six families for both species were plated. The same number of zygotic embryos was plated in the second lot. A total of 600 JP zygotic embryos (JPZE) and 600 BS zygotic embryos (BSZE) were plated.

The formula for Modified Litvay medium (for induction, maturation and germination) used in this study was provided courtesy of Dr. K. Klimaszewska.

Table 3-1. Modified Litvay medium (Klimaszewska *et al.* 2001)

Components	Induction Medium (mg/L)	Maturation Medium (mg/L)		Germination Medium (mg/L)
		6%Sucrose	3.4%Sucrose	
NH_4NO_3	825	825	825	413
KNO_3	950	950	950	950
MgSO4.7H20	925	925	925	463
KH_2PO_4	170	170	170	170
$CaCl_2.2H_20$	11	11	11	5.5
KI	2	2	2	1
H_3BO_3	15	15	15	7.5
$MnSO_4.H_20$	10	10	10	5
$ZnSO_4.7H_20$	21	21	21	10.5
$Na_2MoO_4.2H_2O$	0.6	0.6	0.6	0.3
$CuSO_4.5H_2O$	0.25	0.25	0.25	0.125
$CoCl_2.6H_2O$	0.065	0.065	0.065	0.03
$FeSO_4.7H_2O$	13.9	13.9	13.9	7.0
Na_2EDTA	18.7	18.7	18.7	9.3
Myoinositol	100	100	100	50
Niacinamide	0.5	0.5	0.5	0.25
Pyridoxine.HCl	0.1	0.1	0.1	0.05
Thiamine.HCl	0.1	0.1	0.1	0.05
Sucrose	10 000	60 000	34 000	20 000
Agar	9 000			
Gelrite		4 000	4 000	4 000
Casein Hydrolysate	1 000	1 000	1 000	500
Glutamine (filter-sterilized)	500	500	500	500
BAP	1.1			
Kinetin	1.1			
2,4-D	2.2			
ABA		106	106	

2.23 Maintenance of jack pine and black spruce embryogenic tissue

When the first embryogenic tissue (ET) became visible as a white fluffy mass after four weeks it was separated from the compact non-embryogenic tissue (NET) of the callus (Figure 3-1a and Figure 3-1b) using a sharp sterile scalpel and transferred to a fresh induction medium. The tissue was subcultured onto fresh medium every two weeks thereafter for eight more weeks. The

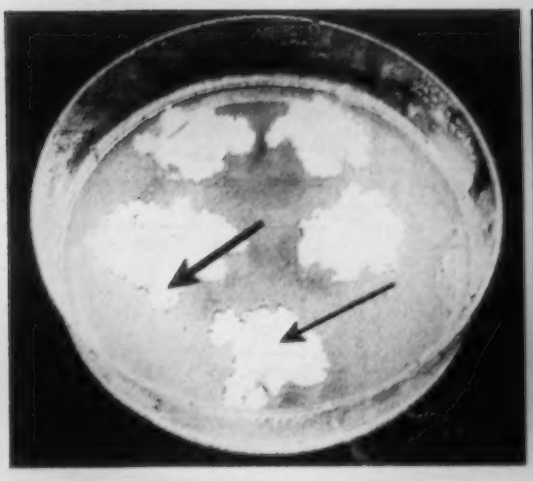


Figure 3-1a. Jack pine embryogenic (thick arrow) and non-embryogenic (thin arrow) tissues

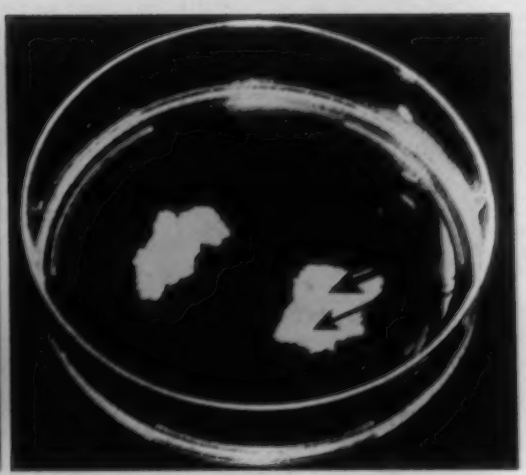


Figure 3-1b. Black spruce embryogenic (thick arrow) and non-embryogenic (thin arrow) tissues

Figure 3-1c. Jack pine Type I embryogenic calli

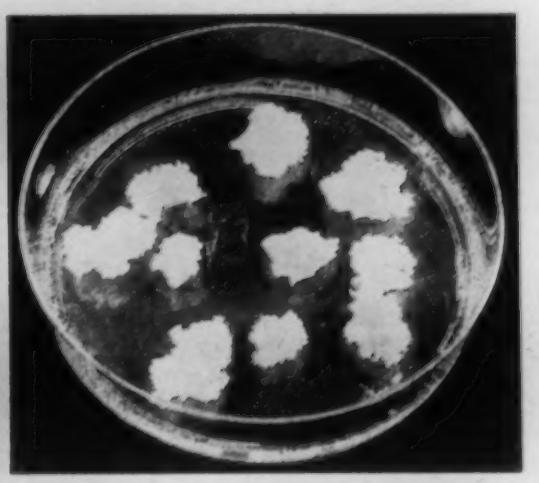


Figure 3-1d. Jack pine Type II embryogenic calli

number of subcultures was limited to prevent the occurrence of somaclonal variation (Winicov 1994). Large SE tissue masses (approximately five grams and more) were cut in half at each subculture. The petri plates were sealed with parafilm and kept in the dark at 25°C for two weeks. After eight weeks, cell lines with three grams or more of the tissue were considered for treatment with sodium chloride. One-third of their tissue mass was placed in pre-maturation media to continue the process of SE. The remaining 2/3 of the tissue mass was divided into four parts and placed in four 100 X 25 mm petri dishes (Fisher Scientific) of pre-germination screening media [induction media plus select concentrations of NaCl (Sigma-Aldrich): 0% (0 mM), 0.5% (85.51 mM), 0.7% (119.72 mM), 1.0% (171.03 mM).

2.24 Maturation into emblings

All the JP and BS cell lines that were transferred in pre-maturation media were kept on the same type of media for three weeks, with one subculture after two weeks. Seven days after the last subculture, nine clumps of healthy tissue (50-100 mg pieces) from each cell line were transferred onto a maturation medium in 100 X 25 mm petri dish (Fisher Scientific). The dishes were sealed with parafilm and placed in 75% screened fluorescent light for 16 hours at 25°C. Production of mature somatic embryos commenced after four weeks and went on for another two weeks.

The mature embryos were transferred to 200 µm nylon mesh discs and placed in 100 X 25 mm petri dish (Fisher Scientific) containing maturation medium to undergo pretreatment by desiccation. The petri dishes were sealed with parafilm and placed in the dark for 2 days at 4°C. Then, the discs containing the embryos were transferred to dry wells in a six-well multi dish tissue culture plate (Falcon). Three discs were placed in three separate wells, and 5-ml sterile water was placed in each of the three empty wells. The culture plates were sealed with parafilm and covered with aluminum foil for seven days at 4°C.

Mature somatic embryos with well-formed cotyledons were chosen and placed in germination medium (Figure 3-2c and 3-2d). The petri dishes were placed in a dark growth chamber for seven days at 25°C. The dishes were then exposed to the screened light at 25°C until the developing root was about five-mm long, or about two weeks on the germination medium.

A potting mix containing pasteurized soil, peat moss and vermiculite (3:1:1) was prepared in planting trays. The potting mix was thoroughly moistened by soaking the trays in water. The roots of the germinated embryos were carefully washed with distilled water to remove any sucrose that may cause fungal growth and rotting. Using a spatula, tiny holes were dug into the potting mix and the emblings were directly transferred to the planting trays. The trays were covered with plastic, and were placed in the greenhouse for acclimatization.

2.25 Treatment with sodium chloride and tissue harvest

For cell lines that were able to produce at least three grams of tissue after bulking for six weeks, two-thirds of their mass was divided into four. The four pieces were transferred into four plates containing induction medium with different sodium chloride (NaCl) concentrations .The plates were labeled C, T1, T2 and T3 for control, Treatment 1, Treatment 2, and Treatment 3 respectively. Control plates contained 0% NaCl, T1 plates contained 0.5% (85.51 mM) NaCl, T2 plates contain 0.7% (119.72 mM) NaCl and T3 plates contain 1.0% (171.03 mM) NaCl.

The tissues were subcultured every two weeks onto fresh induction media containing the same amounts of NaCl for six weeks. Variants that are not stable, like those, which arose through epigenetic changes, will less likely maintain their tolerance in the absence of selective pressure by NaCl (Winicov 1994). To identify stable variants, tissues that survived the salt treatment for six weeks were subcultured onto control medium for six weeks and were placed back into the treatment media for two weeks. Cell lines that are stably salt tolerant do not lose their ability to

Figure 3-2a. Jack pine Type I embryogenic tissue

Figure 3-2b. Black spruce Type II embryogenic tissue

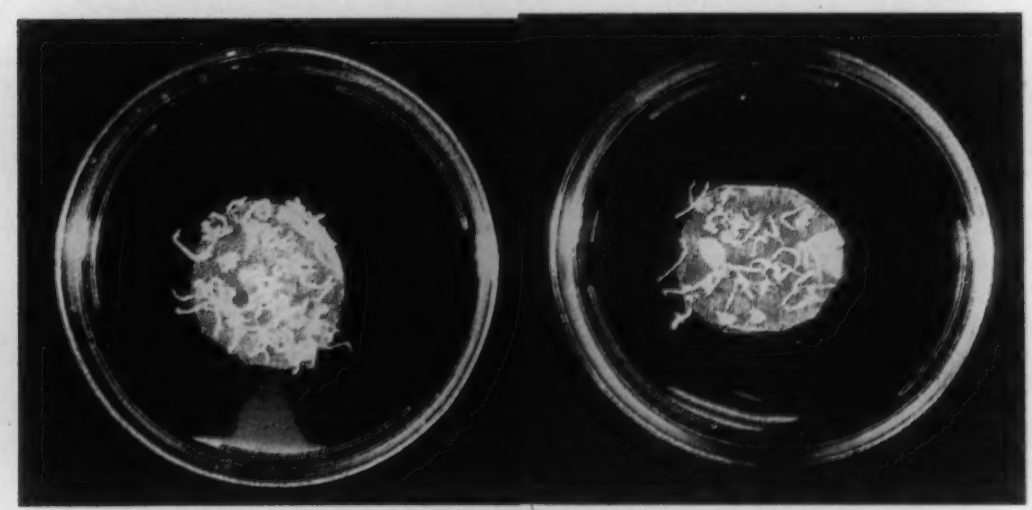


Figure 3-2c. Jack pine mature somatic embryos

Figure 3-2d. Black spruce mature somatic embryos

grow on a high level of salt after subculture on control medium (Winicov 1994). This step should screen out tissues that survived as a result of epigenetic changes. Also calli was used in this study because it was reported that salt selection using callus is likely to introduce fewer genetic rearrangement (Winicov 1994).

Tissues from clones in the control or in any of the treatment plates with at least 50-100% increase in weight after six weeks of treatment were considered priority samples for DD RT-PCR. For every cell line, percent increase in live weight was measured as the difference between the initial weight (W_i) and the final weight (W_f) of the clones after six weeks of treatment divided by (Wi) X 100% The plate of medium to which the clones was first subcultured after bulking was pre-weighed (W_{pl}) then the clones were transferred into it and the plate with the clones was weighed again ($W_{pl+clones}$). The initial weight of the clones (Wi) is calculated by subtracting W_{pl} from $W_{pl+clones}$. On the last subculture, the weight of the clones was calculated in the same way $W_{clones} = W_{pl+clones\ (i)} - W_{pl}$ and at the end of the last two weeks, the plates with the clones were weighed again and recorded as $W_{pl+clones(f)}$. The weight of the clones final (Wf) is calculated by subtracting $W_{pl+clones(i)} - W_{pl+clones(f)}$. Tissues from control and treated plates were harvested by scooping them with a sterile spatula and transferring to a labeled aluminum foil pouch. The pouches were immediately immersed in liquid nitrogen for about two to five minutes, and transferred to an ultra freezer (-80°C, Forma Scientific).

2.3. Tissues from organogenesis of jack pine and black spruce (*in vitro*)

Harry and Thorpe (1994) have demonstrated organogenesis of jack pine from mature embryos in the past. The principles behind their protocols were basically the same as the ones used in this study. One hundred seeds from each family of jack pine (total of 600 seeds) and 100 seeds from each family of black spruce (total of 600 seeds) were surface-sterilized and soaked in double distilled deionized water overnight. The 100 seeds per family were randomly plated in twenty 100

X 25 mm petri dish containing ½ MS medium (Sigma) (Per liter: 4.57 grams MS Basal, 4 grams MS organics, 30 grams of sucrose, and 2.7 grams of agar) supplemented with 0.5 uM 1-Napthalene Acetic Acid (NAA) and 1 uM 6-Benzylaminopurine (BAP). These 20 plates contain varying NaCl concentrations 0% (0.00mM), 0.5%(85.51 mM), 0.7%NaCl (119.72 mM) and 1.0% (171.03 mM) NaCl. Five seed were plated in each plate. In this part of the study, selective media (½ MS with varying salt concentrations) was used to discriminate tolerant from non-tolerant individuals at the germination stage up to six weeks old.

The explants were subcultured on the same medium every three weeks. After six weeks, they were transferred onto a rooting medium (with no NaCl) containing two uM 1-napthalene acetic acid (NAA) (Sigma) until roots and shoots were developed (about three weeks). To screen for stable variants; the seedlings were transferred to control media for six weeks and then returned to selective media for another two weeks. At the end of treatment, for every family of jack pine, and black spruce, five to seven seedlings were pooled together from each treatment to make a sample for DD-RT-PCR.

2.4 Tissues of jack pine seedlings in soil

Dr. Charles Tauer, Professor in Forest Genetics at Oklahoma State University, generously provided the seedlings used in this part of the study. The seed were collected from an Eastern Manitoba jack pine seed zone and were planted July 6, 2000 at the University of Alberta Greenhouse. There were 74 pots each containing 4.5-months-old jack pine seedling. The pots were numbered one to seventy-four. The first 24 pots belong to family 565 while the remaining 50 pots belong to family 111. The pots were divided into treatment groups. Since the pots were already arranged in columns of five (except for the second column of Family 565 which had only 4 pots), the first column belonging to Family 565 was labeled control A, and the subsequent columns control B, T1, T2 and T3. The fifty pots of Family 111 were divided into five of two

columns each. The first division was labeled control A, and the subsequent divisions control B, T1, T2 and T3. Heights of plants were measured starting from the base to the apex of the seedling and recorded as initial heights.

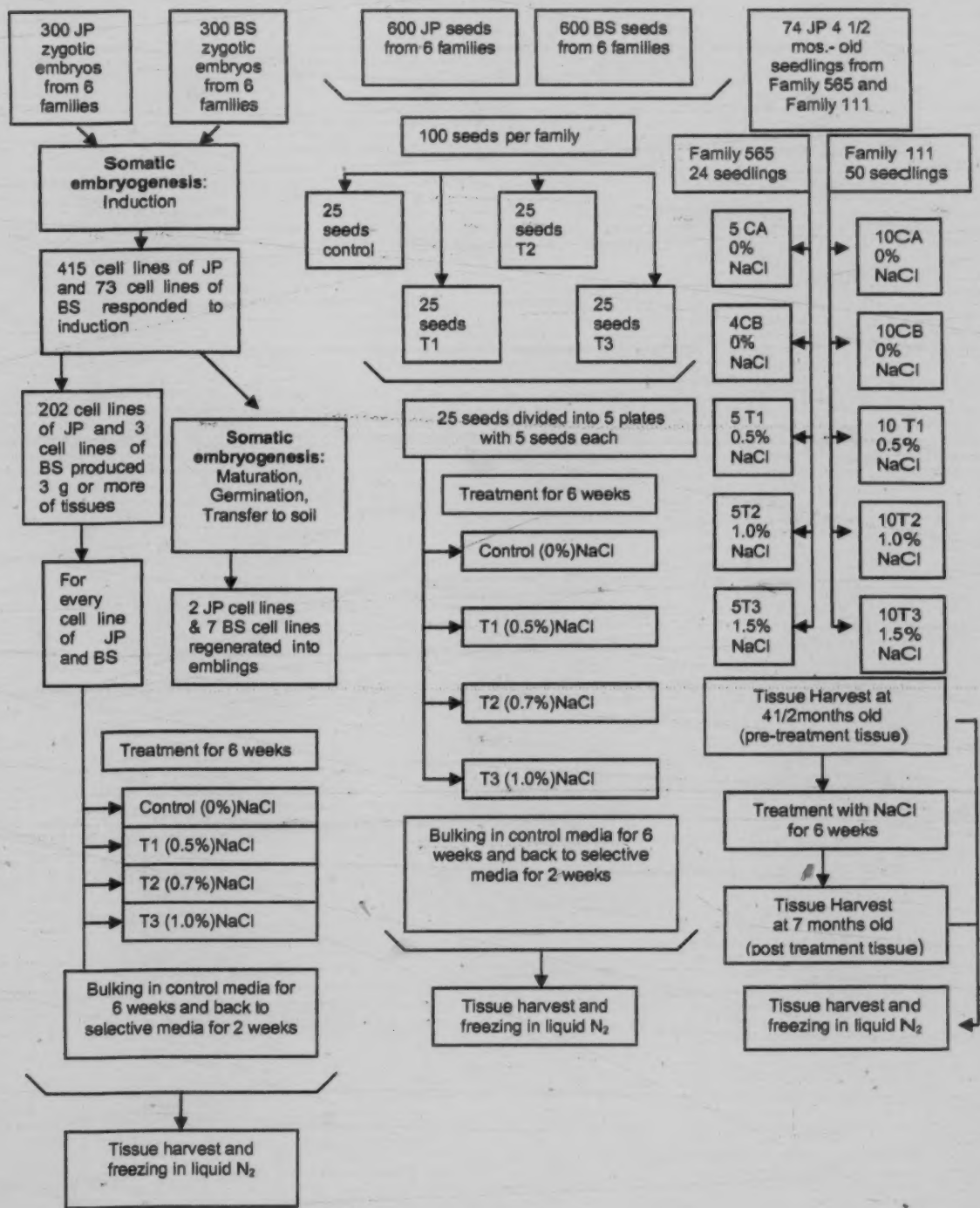
About five grams of mature needles were harvested from each plant except those in control A, and placed in separate 50 ml conical plastic tubes labeled with the family, pot number and treatment, and the weight of the tissue. These tissues were labeled "pre-treatment" (Pr) and were kept in an -80°C-freezer (Forma Scientific) until processed for RNA extraction. Although salt tolerance results from the integration of numerous physiological processes as well as structural adaptations, evidence suggests that enhanced ability to exclude Na^+ and Cl^- from roots or shoots is the most important mechanism operating in salt-tolerant lines of woody species (Allen et al. 1994). This therefore implies that major genes involved in salt tolerance may be found in shoots or roots hence, the reason for using shoots as main source-tissue for RNA extraction.

Under the same set of greenhouse conditions originally recommended by Dr. Tauer (21°C daytime temperature and 19°C night time temperature, average humidity of 25%, 16 hours of light using High Intensity Sodium Bulbs) the plants were treated with varying concentrations of NaCl, 0% for controls, 0.5% (85.51 mM) for T1, (171.03 mM) for T2 and 1.5% (256.54 mM) for T3. Sodium chloride (Sigma) was dissolved in water, and 250 ml of this salt solution was used to water the plants every Tuesday and Friday for six weeks starting December 7, 2000. Every other Friday, the plants were fertilized with 30-10-10 Ever Acid Fertilizer (Plant Products™) one gram/L. This was the same fertilizer used by Dr Tauer to fertilize these plants after germination to about four-and-a-half-months old. Whenever the plants were fertilized, the NaCl crystals were added to the fertilizer solution. The pots were well spaced to ensure that solutions from one pot did not cross contaminate the neighboring pots. Care was taken to wet only the soil. The salt was thoroughly dissolved before using the solution, and each pot received equal amounts of water each time. Salt tolerance was assessed as plant survival and the ability to retain green needle

tissue under saline conditions. At the end of six weeks, when the plants were seven months old, they were observed for their final external appearance, and heights were measured and recorded as H_f or height final. Those plants that were extremely dehydrated, withered, and yellowed were discarded. Those with at least 50% green needles were uprooted. The roots were cleaned with running water. The young needles were wrapped in separate aluminum foil from that of the mature needles, and the roots. Each sample was weighed and the wrappers were labeled with the sample identification code (family, treatments and pot number), the part (YL for young leaves, ML, for mature leaves, R for roots) and its weight in grams. Each plant part was placed in separate plastic bags, and all these tissues were labeled "Post-treatment"(Pt). The tissues were all placed in -80°C-freezer until processed for RNA extraction.

Diagram 3-1 on the following page summarizes the entire screening procedure.

Diagram 3-1. Summary of the Screening for Salt Stress Responses in Jack Pine and Black Spruce


300 JP zygotic embryos from 6 families
300 BS zygotic embryos from 6 families
Somatic embryogenesis: Induction
415 cell lines of JP and 73 cell lines of BS responded to induction
202 cell lines of JP and 3 cell lines of BS produced 3 g or more of tissues
Somatic embryogenesis: Maturation, Germination, Transfer to soil
For every cell line of JP and BS
2 JP cell lines & 7 BS cell lines regenerated into emblings
Treatment for 6 weeks
Control (0%)NaCl
T1 (0.5%)NaCl
T2 (0.7%)NaCl
T3 (1.0%)NaCl
Bulking in control media for 6 weeks and back to selective media for 2 weeks
Tissue harvest and freezing in liquid N2
600 JP seeds from 6 families
600 BS seeds from 6 families
100 seeds per family
25 seeds control
25 seeds T1
25 seeds T2
25 seeds T3
25 seeds divided into 5 plates with 5 seeds each
Treatment for 6 weeks
Control (0%)NaCl
T1 (0.5%)NaCl
T2 (0.7%)NaCl
T3 (1.0%)NaCl
Bulking in control media for 6 weeks and back to selective media for 2 weeks
Tissue harvest and freezing in liquid N2
74 JP 4 1/2 mos.- old seedlings from Family 565 and Family 111
Family 565 24 seedlings
Family 111 50 seedlings
5 CA 0% NaCl
4CB 0% NaCl
5 T1 0.5% NaCl
5T2 1.0% NaCl
5T3 1.5% NaCl
10CA 0% NaCl
10CB 0% NaCl
10 T1 0.5% NaCl
10T2 1.0% NaCl
10T3 1.5% NaCl
Tissue Harvest at 41/2months old (pre-treatment tissue)
Treatment with NaCl for 6 weeks
Tissue Harvest at 7 months old (post treatment tissue)
Tissue harvest and freezing in liquid N2

3. Results and Discussion

3.1 Somatic embryogenesis of jack pine and black spruce

Four weeks after plating, the dissected zygotic embryos, started to swell. They produced a transparent, viscid liquid that resembled spider webs when stretched. The white opaque zygotic embryos became light brown, very soft, and were starting to lose their original elongated shape to an amorphous structure. Toward the end of the first subculture, all shape was lost. The cultured embryos appeared as just a mass of light brown tissue surrounded by white clear viscid fluid. They were about twice their original size. Only the cotyledons of some zygotic embryos proliferated, some only the radicle-end, while some both ends including the hypocotyl. Within the jack pine species, individual cell lines showed a slightly different response in terms of embryogenic tissue (ET) they produced. After two months on induction media, some embryos produced soft, bright lemon yellow tissues that tended to remain close together, while others produced a dull yellow, hard, and more compact tissue mass (Figure 3-1c and 3-1d). Most of the black spruce zygotic embryos developed hard, non-embryogenic calli. At first the embryogenic calli was light yellow, then it became pale brown and then dark brown. Those that responded to induction developed a white loosely packed mass of cells that looked like miniature" cauliflower". (Figure 3-2b). It was observed in both JP and BS that even after the 4th subculture, the brown tissues continued to produce embryogenic tissues. After the 5th subculture, some of the tissue masses in both species became rust-brown. The tissues clumped and were soft, some were a little bit dry, and they appeared to have lost their embryogenic characteristic. In the first lot of zygotic embryos (ZE), 91.67% (275/ 300) of JP and 22.33% (67/300) of black spruce produced somatic embryos after one month of induction. In the second lot of zygotic embryos, 48.67% (146/300) of JP and only 1% (3/300) of BS produced somatic embryos after one month of induction.

Table 3-2 Production of embryogenic tissue among the first and second lot of zygotic embryos after two and a half months in induction media

First Lot of Zygotic Embryos		
Family	Jack Pine	Black Spruce
1	100% (50/50)	30% (15/50)
2	100% (50/50)	14% (7/50)
3	100% (50/50)	20% (10/50)
4	100% (50/50)	24% (12/50)
5	100% (50/50)	2% (1/50)
6	78% (39/50)	50% (25/50)
Second Lot of Zygotic Embryos		
1	38% (9/50)	2% (1/50)
2	46% (23/50)	0% (0/50)
3	62% (31/50)	0% (0/50)
4	64% (32/50)	4% (2/50)
5	52% (26/50)	0% (0/50)
6	10% (5/50)	0% (0/50)

In the first lot of zygotic embryos, 96.33% (289/300) of the JP and 23.33% (70/300) of the BS produced embryogenic tissues. In the second lot, 42% (126/300) of the jack pine and 1% (3/300) of the black spruce produced embryogenic tissues. It is possible that the big difference in the response between the first and second lot of zygotic embryos could be attributed to age of explants from the time of collection and storage to the time of plating as well as the species themselves. The seeds used in the second lot were stored longer than the first lot before they were actually plated onto the induction media. It was also notable that it took a longer time to induce black spruce zygotic embryos (BSZE) to develop embryogenic tissue than jack pine zygotic embryo (JPZE). This could be explained by the fact that generally, black spruce grows more slowly than jack pine. Overall 69.17% (415/600) of the JPZE and 12.17% (73/600) of the BSZE produced embryogenic tissues. From the results above, intraspecific variation is notable in both black spruce and jack pine.

The kind of embryogenic tissue produced per cell line varies from one cell line to another and within families. Except for very few cell lines that produced Type II embryogenic calli, jack pine produced a Type I embryogenic calli that looked organized, compact, pale yellow in color and

slow growing (Figure 3-1d and Figure 3-2a). Black spruce produced Type II embryogenic calli that were friable, soft, and somewhat translucent and rapidly growing (Figure 3-1b and Figure 3-2b). The embryogenic tissues produced by black spruce were white, and fluffy or less compact.

Of the 415 cell lines of jack pine and 73 cell lines of black spruce that responded to induction, only 7 BS cell lines and 2 JP cell lines were able to produce somatic embryos and regenerate into plantlets (BS1-3 cell line; BS2-1 cell line; BS3-1 cell line; BS4-1 cell line; BS6-1 cell line and two cell lines from JP 4 (Figures 3-2c and 3-2d). These 9 cell line somatic embryos successfully germinate and fully developed roots and shoots and were transferred to the soil. This low regeneration observed for JP and BS supports the findings of the Canadian Forest Service in their study of somatic embryogenesis of pines and spruces. Although the percentage of cell lines that reached maturation was relatively low, the number of mature embryos per cell line was relatively high, about 100 or more in less than a gram of embryogenic tissue.

Of the 415 cell lines of JP and 73 cell lines of BS that responded to induction only 202 cell lines of JP and 3 cell lines of BS were able to produce three grams or more of the tissue. Only these 202 cell lines of JP and 3 cell lines of BS were considered for treatment with NaCl to screen for salt tolerance. About 4% of the entire cell lines included in the screening for salt tolerance were contaminated with fungi and were discarded. Some cell lines slowed their growth towards the fourth subculture on selection media. Those that originally had a lesser amount of embryogenic tissue in proportion to the non-embryogenic tissues became totally non-embryogenic, and some eventually became necrotic. Figures 3-3a to 3-3d show responses of JP embryogenic tissue to varying NaCl concentrations.

3.2 Organogenesis of jack pine and black spruce in vitro

All of the jack pine seeds in media supplemented with 0.5%, (85.51 mM), 0.7% (119.72 mM),

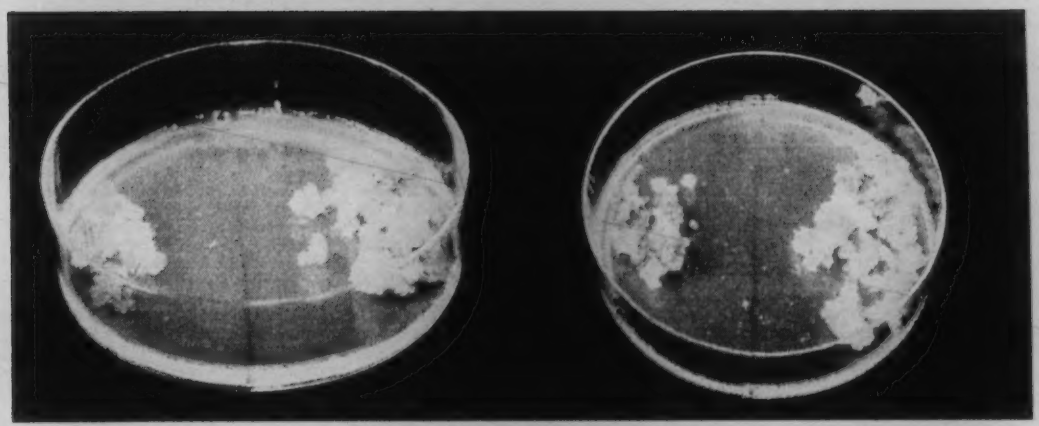


Figure 3-3a. Jack pine cell line 87 (left) and cell line 113 (right) belonging to family 4 in control media (with 0 % NaCl)

Figure3-3b. Jack pine cell line 87 (left) and cell line 113 (right) belonging to family 4 in selective media (with 0.5% NaCl)

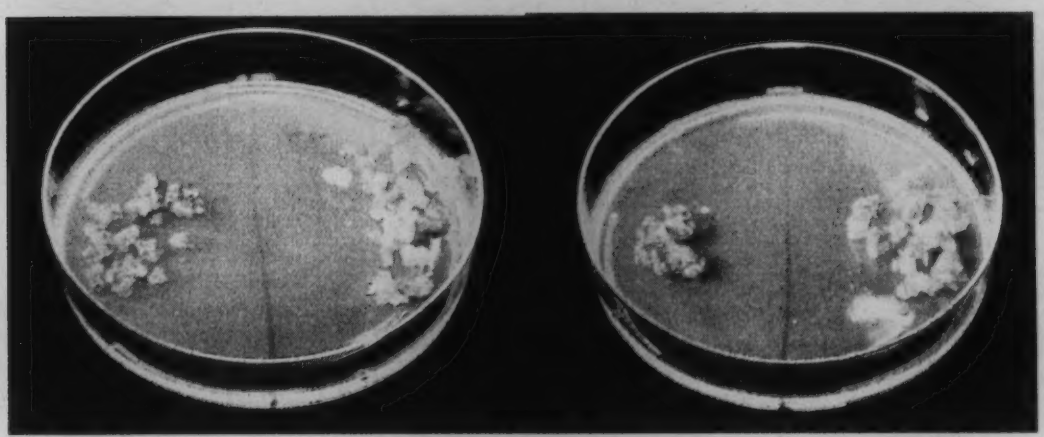


Figure 3-3c. Jack pine cell line 87 (left) and cell line 113 (right) belonging to family 4 in selective media (with 0.7% NaCl)

Figure 3-3d. Jack pine cell line 87 (left) and cell line 113 (right) belonging to family 4 in selective media (with 1.0% NaCl)

1.0% (171.03 mM) NaCl germinated as those in control media. However, it took a much longer time for the seeds to germinate on the solid selective media containing NaCl compared to seeds in control media. After a week on the selective media, the radicle grew very slowly, and the length of the developing radicle appears to be related to the level of salt concentration in the media: the higher the concentration the shorter the radicle.

For a reason that cannot be explained, all of the seedlings on 0.7 % NaCl didn't grow any further after the seed coat broke open and the radicle started to protrude. It seems that their growth was arrested shortly after the radicle came in contact with the salty media. The total weight of germinated seeds was too small to be considered sufficient to make a sample for RNA extraction and therefore all of the germinated seedlings treated with 0.7% NaCl were not considered for RNA extraction.

Black spruce growth was very slow compared to jack pine. After six weeks, the amount of BS tissue was far smaller than the amount of JP tissue produced on the control plates. All of the six JP families were able to survive 0.5% and 1.0 % NaCl treatment compared to only one family of black spruce. BS was found to be generally more sensitive to salt stress than JP.

3.3 Jack pine seedlings in soil

Higher NaCl concentrations (1.0 and 1.5% for T2 and T3 respectively) were used for the older potted seedlings following the idea that salt tolerance may be developed with age. Higher salt concentrations may be required to discriminate tolerant from non-tolerant individuals at an older stage of seedling development.

One of the mechanisms involved in surviving salt stress known in plants is the sequestration of ions into the mature leaves, specifically the older ones, sparing the young meristematic tissues. Observations of the seedlings after six weeks of treatment seem to imply that the same

mechanism works in jack pine seven-month-old seedlings. In the treated seedlings, the signs of salt toxicity were observed in the older leaves such as yellowing of the needles, which became severely dehydrated and eventually dehisced. This observed difference between young and old needles was more severe among T3 plants treated with 1.5% (256.54mM) NaCl. The responses observed among the clones generated by SE, the seedlings germinated in vitro, and the seven-month-old seedlings were consistently the same, with the older needles showing the signs of toxicity first. The severity of their symptoms also appeared to be positively related with the concentration of salt in their substrate: the higher the NaCl concentration the more severe the symptoms.

As of January 8, 2001, after more than a month of treatment, five out of five and ten out of ten T3 plants in both families showed severe signs of toxicity; yellowing of leaves, dehydration, and necrotization of needle tips. Three out of five (Family 565) and six out of ten (Family 111) T2 plants showed the same signs but more than 50-75% of their tissues remained green and healthy. About 10% of the T1 plants (one for each family) showed milder symptoms of toxicity such as slight browning of the needle tips, but the rest of the plant remained green and healthy. All the controls from both families did not show any signs of toxicity.

When final heights and initial heights were compared there was a decreasing growth trend as concentration of sodium chloride increased. All individuals in both families treated with 0.5% NaCl solution showed tolerance after 6 weeks of treatment, with tissues of all 15 plants at least 85%-100% green. None of the individuals of either family in T3 survived the treatment after six weeks. The needles were brittle and fell off from the stem. In all T3 and T2 plants with only 20% or less of their tissues remaining green after six weeks of treatment, it was consistently notable that their youngest needles were the last to show the symptoms of toxicity. They young tissues always appeared green even when all the mature needles were starting to fell off from the stem.

There was great variability in salt tolerance among individuals within the same family of JP and BS. The maximum concentration of NaCl that could be tolerated by most JP and BS seedlings in this study was 0.5% (85.51mM). There were 96/600 explants of JP seedlings and 18/600 explants of black spruce seedlings in vitro that survived 0.5% NaCl. At the callus stage, there were 120/244 (49%) cell lines of JP embryogenic tissue (JPET) with at least 50% of normal growth at 0.5% NaCl. None of the black spruce embryogenic tissue (BSET) had even 1% of normal growth at the same 0.5% NaCl concentration. BSET are generally more sensitive to salt stress than JPET. Also there seems to be a higher tolerance exhibited by JP embryogenic tissues than the seven-month-old seedlings, as many of the embryogenic tissue survived the T3 treatment (1.0% or 171.03 mM NaCl) compared to few survivors among the seven-month-old seedlings in T2 treatment (1.0% or 171.03 mM NaCl). There were 68/244(27.87%) JP cell lines that showed at least 1% growth after T3 treatment while 1/15 (6.67%) of the seven-months-old seedlings were able to retain at least 85-100% green tissue after the treatment using same NaCl concentration. On the contrary, seven-month-old JP seedlings were more tolerant than seedlings treated at germination *in vitro*. At 0.5% NaCl, there were 96/600 JP seedlings *in vitro* that survived the treatment compared to 15/15 JP in soil that survived the same NaCl concentration. These results support the reports of others reviewed that seeds of many plants have relatively higher tolerance than their very young seedlings, and that tolerance developed with age. It also suggests that as in tomatoes (Foolad, 1996) and wheat (Mano & Kayzuyoshi, 1997), different genes for salt tolerance may be working at different stages of development in JP and BS, hence the observed differences in sensitivity among clones, newly germinated seeds and seven-months-old seedlings.

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CHAPTER IV

IDENTIFICATION, ISOLATION, AND CHARACTERIZATION OF TRANSCRIPTS RELATED TO SALT STRESS RESPONSE

1. Introduction

Transcripts related to salt stress have already been isolated and identified in such organisms as tomatoes, arabidopsis, algae, and yeast. None, however, were reported to have been isolated from forest trees, specifically from pines or spruce. To address this deficiency, this study focused on two common forest tree species jack pine and black spruce. Using DD RT-PCR transcripts that were both up-regulated and down-regulated in response to NaCl stress were isolated from embryogenic tissue with surrounding calli, seedlings germinated and treated in vitro, and from seven-month-old seedlings treated in soil.

When this study was started in 1999, there were other techniques available for studying differentially expressed genes and to quantify their expression such as microarray and real-time PCR. These techniques however, were not yet perfected and are still very much in the process of development. GenHunter Corporation reported 1545 papers already published using Differential Display (DD) based on a Medline publication search. Considering its reported enormous popularity and success in detecting alterations in gene expression in diverse biological systems, it was considered a dependable method (Luca 2001) for this study.

DD uses a limited number of short arbitrary primers in combination with anchored oligo-dT primers to systematically amplify and visualize most of the mRNA in a cell. The conditions, quality of RNA samples, primers, MMLV RT, oligo-dTs and arbitrary primer combination, Taq polymerase, and PCR tubes used were optimized to maximize the number of bands amplified, yield strong signals, and show good reproducibility. Gen Hunter Corporation has reported that the

use of short arbitrary primers and anchored oligo-dT primers are preferred for PCR to specifically amplify a target DNA sequence. Longer primers are used to amplify specific genes, while shorter primers are used to amplify multiple genes (GenHunter Corporation 1996-2002). The one-based oligo-dT anchored primers were shown to be much more efficient for DD. Thirty arbitrary primers in the GenHunter RNA image® Kit in combination with each of the three one-base anchored primer were screened to determine the primer combinations that would amplify the most RNA, give the strongest signals and be reproducible. The RNA image kit features the 3rd generation of Differential Display technology using three one-base-anchored oligo-dT primers. This reduces redundancy and potential under-representation of the subpopulation of mRNAs (GenHunter Corporation 1997).

2. Materials and Methods

2.1 Total RNA extraction

For JP and BS embryogenic tissues and its surrounding calli, 100-200 mg from each of the control and treated tissues were sampled for every cell line. Priorities are those samples with 100% increase in weight after six weeks of treatment. For seedlings germinated in vitro, each sample of approximately 100-200 mg was a pool of five to seven individual plants from the same family and from the same treatment and petri dish. For the seven-months-old JP seedlings in soil, every sample pair of "control" and "treated" were needles and some parts of the stem from pre treated and post treated tissues of the same individual respectively.

Dr. Yinghua Huang shared an unpublished protocol for RNA and DNA extraction through his former supervisor Dr. Charles Tauer of Oklahoma State University. The protocol was modified to optimize conditions for the samples used in this study. Modifications from the original Yinghua

Huang protocol were that second day of centrifugation at 10 000 rpm for two hours was changed to 15 000 rpm for 30 minutes and centrifugation following chloroform-isoamyl extraction was changed from 10 000 to 12 000 rpm.

All glassware, spatulas, mortar and pestles used in this step were baked overnight. Micropipette tips and water were DEPC treated and autoclaved. Newly opened RNAse-free 1.5-ml eppendorf and oakridge tubes were also autoclaved prior to use. The following is the modified Yinghua Huang Protocol for RNA extraction. For every cell line, control and treated tissues were grounded separately in liquid nitrogen using mortar and pestle. For every 0.1 to 0.2 grams sample, 1.6 ml of Extraction Buffer (2% CTAB, 2%PVP, 100mM Tris-HCL, pH 8.0, 25mMEDTA, 2.0M NaCl, 0.5g/lspermidine) with 32 µl of mercaptoethanol was warmed to 65°C in a water bath and added to the frozen grounded tissues. The samples were incubated at in a 65°C water bath for exactly 15 minutes. An equal volume of chloroform-isoamyl alcohol (24:1) was added to each and the mixtures were vortexed for 10 seconds. The extraction step with chloroform-isoamyl was repeated twice, followed each time by centrifugation at 12 000 rpm for 10 minutes at room temperature and collection of the clear aqueous phase. After the second extraction, 1/4-1/3 volumes of 10M LiCl was added to each supernatant, and the RNA was allowed to precipitate overnight at 4°C. After the overnight precipitation with LiCl, the samples were centrifuged at 15 000 rpm for 30 minutes at 4°C. The supernatants were decanted, and the pellets washed with 500 µl of SSTE buffer (0.6M NaCl, 0.2%SDS, 10mM Tris-HCL, 1mMEDTA, pH8.0, autoclaved). An equal volume of chloroform-isoamyl was added to each and the contents were thoroughly mixed by vortexing for 10 seconds. The samples were centrifuged at 12 000 rpm for 10 minutes at room temperature, followed by the collection of the clear aqueous phase. The supernatants were placed in new 1.5-ml eppendorf tube, and 2X volume of 100% ethanol was added. The samples were placed in a-80°C freezer to precipitate the RNA for two hours. After precipitation, the samples were centrifuged for the last time at 15 000 rpm for 30 minutes. The supernatants were decanted, and the RNA pellets were air dried for approximately 15-30 minutes. The RNA

pellets were resuspended in 25-50 µl of DEPC-treated water. Five µl of the suspended RNA was used for quantification using the GeneQuant (Pharmacia-LKB) while the remaining samples were stored in the-80°C freezer until further use.

For larger samples like the needles from JP seedlings in soil, the volumes of the reagents were proportionately increased with the amount of tissue processed, and oakridge tubes were used in lieu of 2-ml tubes. The larger samples were spun in SS-34 rotor Sorvall RC-5B Refrigerated Super Speed Centrifuge (Du Pont Instrument, Mandel Scientific Company Ltd.) at 4°C.

Total RNA was extracted from:

1) 12 samples of pooled jack pine seedlings germinated and grown in control and selective media (six controls and six treated): JPIC, JP1T, JP2C, JP2T, JP3C, JP3T, JP4C, JP4T, JP 5C, JP5T, JP6C, JP6T;
2) Six samples of pooled black spruce seedlings germinated in vitro grown in control and selective media (three controls and three treated: BS 2C,BS 2T, BS 5C, BS 5T, BS 6C, BS 6T;
3) 40 samples of jack pine embryogenic tissue with surrounding calli: sample code 134C &T3, 138C&T3, 139C&T3, 103C&T3, 128C&T3, 232C&T3, 107C&T3, 244C&T3, 3C&T3, 1C&T1, 31C&T3, 205C&T3, 216C&T, 97C&T3, 84C&T2, 88C&T3, 100C&T3, 129C&T2, 130C&T3; 131C&T3;
4) two samples of black spruce embryogenic tissue with surrounding calli: sample 32C&T1;
5) 12 samples of seven-months-old jack pine seedlings in soil: CA- 25(111); T2- 55(111), T1-10 (565); CB-7 (565); T1-46 (111), CB-35 (111) post treatment and pre-treatment tissues.

Only extracted total RNA samples with the relatively best properties in terms of absorbance (0.025 and higher), concentration (at least 0.2 µg /µl), purity (95%-100%), and ratio (1.6-1.9) were included in the Differential Display reactions.

2.2 Differential Display Reverse Transcription-Polymerase Chain Reaction (DD RT-PCR)

2.21 Reverse transcription (RT)

Total RNA samples were run in formamide gels (7% formaldehyde gel: 1.1 g agarose, 83.4 ml DEPC-treated double distilled deionized water, 10 ml 10X MOPS, 7 ml formaldehyde) to observe their quality and integrity. No-RT PCR reactions were performed to check for DNA contamination. Samples with DNA contamination were treated using the DNA-free™ kit (Ambion). Total DNA-free RNAs were diluted to 0.2 µg /µl. Using the GenHunter RNA Image™kit (protocol modified to optimize conditions for the samples), 20 µl of the RT cocktail was prepared per sample by mixing two µl of two µM oligo-dT primer H-T_{11} A, four µl of 5X RT Buffer, 1.6 µl of 250 µM dNTP, 9.4 µl of distilled water, and two µl of diluted total RNA sample (0.2 µg/µl). The anchored oligo-dT primers used contain 11 thymine residues followed at the 3' end by one adenine nucleotide. The RT reactions were catalyzed by MMLV (Moloney Murine Leukemia Virus Reverse Transcriptase) with successive steps of denaturation at 65°C for five minutes; annealing at 37°C for 60 minutes, and extension at 75°C for five minutes. After cooling the RT products to 4°C, they were kept in a -20°C freezer until needed.

2.22 Polymerase Chain Reaction (DD-PCR)

Two µl of the RT product (cDNA) was used in the subsequent PCR reaction. Using GenHunter RNA image Kit, the reaction was carried out using arbitrary primers H-AP_8 along with the same anchored primer H-T_{11}A and infrared labeled dATP to amplify a portion of the cDNA population constructed during the RT reaction. Each PCR reaction contained two µl 10X PCR Buffer, 1.6 µl of 25 µM dNTP, two µl of two µM of H-AP_8 and two µl of two µM H-T_{11}A primer, 0.2 µl of 20 µM infrared-labeled dATP, 0.2 µl of five U/µl of Ampli Taq DNA polymerase (Perkin-Elmer), 10.0 µl of double distilled deionized (ddd) water, and two µl of the RT product (cDNA). The reaction was run

for 40 cycles of denaturation at 94°C for 30 seconds, annealing at 40°C for two minutes, extension at 72°C for 30 seconds, and final extension of 72°C for five minutes. PCR reactions were carried out in Perkin-Elmer 9600 thermocycler. After cooling down the samples to 4°C, the PCR products were kept in the -20°C freezer until needed. The selection of the arbitrary primer H-AP_8 and oligot-dT primer H-T_{11}A was done after screening 30 arbitrary primers (H-AP_1-H-AP_{30}) X three oligo-dT primers (H-T_{11}A, H-T_{11}C, and H-T_{11}G). Other conditions were also optimized, such as the amount of Taq Polymerase, volume of RT products, and annealing temperature.

2.23 Size fractionation

Forty ml of 7% polyacrylamide sequencing gel solution (16.8 g urea, 4.8 ml 10X TBE pH 8.0, six ml 7% Long Ranger acrylamide, double distilled deionized water) with 240 µl of 10% APS and 24 µl of TEMED was prepared. Forty-one-cm plates, 64 tooth shark-tooth comb, and 0.25 mm thickness spacers were used in the preparation of the gel. After two hours of polymerization, the gel plates were mounted onto the scanner, and pre run for 15-20 minutes or until the gel temperature reached 50°C. After pre running, the wells were flushed with 1 X TBE buffer until clear of urea residue. Ten µl of formamide loading/stop buffer [47.5 ml 95% deionized formamide, two ml 0.5M EDTA, 0.5 ml double distilled deionized water, 20 mg Bromophenol Blue (Fisher B-392)] was added to each tube of PCR product and mixed by finger tipping for about five seconds. The samples were denatured at 95°C for three minutes in the thermocycler (Perkin-Elmer 9600) and immediately plunged into ice to rapidly cool. Using the single Hamilton syringe, 0.6 µl of Li-Cor molecular weight marker was loaded into the first and last well. Using the eight-channel Hamilton syringe, 1.2 µl of each sample was loaded into corresponding wells. When the gel was loaded, the upper buffer tank lid was replaced, and the scanner door closed. Following the LiCor procedure, the gel was run for about 8 hours at 1,500 volts, 35.0 ma and 31.5 W, 50°C, scan speed of three, signal filter of three, and pixel size of 16. An image was collected from both the 700 and 800 channel. Images were visualized using the Image Manipulation program V3.00 of

the Sequence BaseImagIR 3.0 (LI-COR, USA). Differences in band pattern between control and treated samples were noted. Bands unique to control or treated sample pairs were identified. The unique bands may represent a difference in mRNA between control and treatment and were considered "differential bands". Band patterns were considered reproducible after three gel runs of the same set of samples gave consistent results. The PCR reaction was repeated using two µl of α-[^{33}P]dATP (2000 Ci/mmole) (Amersham Pharmacia Biotech) and were run in a Perkin Elmer GeneAmp 2400 PCR System using the same set of conditions used in the Perkin Elmer 9600. Each sample was run in duplicate loaded side by side.

2.24 PAGE sequencing and autoradiography

Six percent polyacrylamide gels were prepared to run the P^{33}-labeled samples. The gels were allowed to polymerize for at least two hours. The gels were pre run for 30 minutes and the wells were flushed until clear of urea residues. The P^{33}-labeled samples were denatured for 3 minutes at 95°C in a thermocycler and 1.5 µl of each of the denatured samples were loaded into the corresponding wells. The gels were run using the Vertical Gel Electrophoresis (Tyler Research Instruments) powered by 2297 Macrodrive 5 Constant Power Supply. After running the gel for three and a half hours at constant voltage of 150, the gels were blotted on a piece of 3M Whatman filter paper and covered with Saran wrap. The gels were dried in a Slab Gel Dryer Model SE 1160 (Hoeffer Scientific Instruments) at 80°C for 1.5 hours. Kodak x-ray films were exposed to the gels inside a cassette for about three days at room temperature and were developed for band selection. Duplicate samples gave similar banding patterns, indicating that the amplifications were specific.

2.25. Band selection, extraction of cDNA and reamplification

After developing the above x-ray films, they were reoriented with the gel on a light box. The

orientation was guided by fluorescent strips from Strategene which were placed around the gel before exposure to x-ray films. The bands of interest were located by punching holes around them through the film and the filter paper using a dissecting needle. All the differential bands were excised from the gel with a sterile scalpel and placed separately in sterile 1.5-ml eppendorf tubes (Fisher Scientific). Each of the gel slices along with the 3M paper was soaked in 100 µl of distilled water for 10 minutes. The tube caps were tightly closed with parafilm strips and then boiled for 15 minutes. After boiling, the tubes were spun for two minutes at 14,000 rpm at room temperature to pellet the gel and paper debris. For each tube the supernatant was transferred to a new 1.5 ml eppendorf tube and precipitated with 10 µl of 3M Sodium acetate (NaOAC), five µl of glycogen (10 mg/ml) and 450 µl of 100% ethanol for 30 minutes in an -80 °C-freezer. The DNA was pelleted by spinning for 10 minutes at 4°C and 14 000 rpm. The DNA pellets were washed with 200 µl of ice- cold 100% ethanol. The tubes were spun briefly to remove the residual ethanol. The DNA pellets were air-dried for 15-30 minutes, and dissolved in 10 µl double distilled deionized water. Sixty-six fragments were chosen for cloning and sequencing. Sixteen bands were present in the control but not in the treated, or were very faint in the treated but clearly visible in the control. Forty-seven bands appeared to be present in the treated but not in the control, or very faint in the control but clearly visible in the treated. For each of these 66 fragments, four µl of the recovered cDNA band was used for amplification using the same set of primers and PCR conditions as in the initial differential display PCR reactions, except that the dNTP concentration was 20 µM and no radiolabeled dATP was used. For a 40 µl reamplification reaction per sample, four µl of 10X PCR Buffer, 3.2 µl of 250 µM dNTP, four µl of two µM H-AP_3, four µl of two µM H-$T_{11}A$ primer, 0.4 µl of Perkin-Elmer Taq Polymerase (five U/µl) and four µl of cDNA template were mixed together by briefly vortexing. The remaining six µl of the cDNA samples were saved in a –20°C freezer for future use. Thirty µl of each of the reamplified PCR product was run in a 1.5 % agarose gel and along with four µl of 1Kb Plus DNA molecular weight marker was included (150 µl double distilled deionized water, 50 µl loading dye, 25 µl of the DNA ladder)(Gibco-BRL, 10787-018). Gels were run at 150-mA for 1 hour to visualize bands of the expected size. The cDNA fragments stained with ethidium bromide (10mg/ml) were visualized on

a long wave UV light box and the image was printed using the Molecular Analyst program. The reamplified cDNAs were extracted from the agarose gel using the QIAEX II Gel Extraction Kit (150) (Qiagen Inc., Canada, Catalog No. 20021) and kept in –20°C freezer until needed.

2.3 Bacterial cloning

Bacterial cloning of the select bands of DNA and checking of the insert using the Colony-PCR method were done following the GenHunter PCR-TRAP® cloning system (Cat.No. P404). The results were run in a 1.5% agarose gel stained with ethidium bromide, and viewed under UV light box using the Molecular Analyst Software version 2.1(Bio-Rad Laboratories, Molecular BioScience Group 2000, Alfred Nobel Drive, Hercules, CA 94547). After inserts were confirmed, the source colonies were restreaked onto separate new LB-Tet plates and grown overnight. From each colony plate, a single Tet resistant colony was fished out and inoculated into a 5 ml LB culture (no tetracycline). Three-ml of the LB culture was used for plasmid miniprep and two-ml were used to prepare a 50% glycerol cell stock that was frozen at -80°C. Plasmid minipreps were done using GenElute ™ Plasmid Miniprep Kit (PLN 70, Sigma-Aldrich Corporation).

2.4 Characterization of fragments by sequencing and searching for sequence similarity from databases.

Sequencing was done using Sequiterm EXCEL™ II DNA Sequencing Kit -LC (for 25-41cm gels (Cat. No. SE 9101, Epicentre). This kit was optimized to use with Li COR automated DNA sequencing machines. The GenHunter's Lseq (5' ATC ACG AGG CCC TTT CG 3') and Rseq (5' AACGAA GCA AGC GCAG 3') primers used in this study were fluorescent-labeled by Li Cor. The Cycle Sequencing protocol was followed using 100 femtomoles (fm) of plasmid. The PCR program used was 95°C for 5 minutes (template denaturation), 30 cycles of 95°C for 30 seconds denaturation, 50°C for 15 seconds annealing, and 70°C for one-minute extension.

Homology and similarity among fragments were determined using Gene Jockey II Sequence Processor (Biosoft, Cambridge, UK); the Bionavigator, (Entigen Pty Ltd., Australian Technology Park, Eveleigh NSW 1430) a web-based bioinformatics service; and the Bioedit, (Hall 1997-2001) a biological sequence alignment editor. The sequences of the fragments were also matched mainly with entries in the GenBank and that of the Swiss Institute of Bioinformatics (SIB) using the Basic Local Alignment Search Tool (BLAST®) and the SIB Network Service, respectively. Fragments that were similar to each other were identified and those that were similar to a common gene were pooled together. Gene Specific Primers (GSPs) were designed based on the sequence of the longer fragments, with priority given to those with significant similarity (P or E=0.005) to entries in the GenBank or SIB. Ten GSPs F3, F10, 1C, 5D, F24, F25, F27, F28, F36, F39, were designed using Yahoo's Primer3 software and were synthesized by the University of Alberta DNA Lab.

2.5 Confirmation of expression through RT-PCR

The expression of some of these transcripts was initially confirmed using the Northern Blot Analysis technique. After optimization of conditions and repeated trials, results seem to indicate that most of the RNA transcripts that were isolated are expressed in very low concentration. RT-PCR was chosen as an alternative to the Northern Blot.

Many of the source tissue samples from which these differential fragments were isolated were used up during the Northern Blot Analysis. To confirm expression, total RNA was freshly extracted from nine pairs (control-treated) of embryogenic tissue with surrounding calli. These are JP1Da-7, JP2C-9, JP2E-3, JP3BA002a, JP6E-9, JP4F-12, and JP6E-5, JP4G-1, JP4F-10. These nine RNA samples with acceptable properties in terms of absorbance, ratio, purity, and concentration were selected from 44 samples that were extracted. Using the same protocol used in the DD RT-PCR step, the RNA samples were reverse transcribed. The PCR reaction was optimized with the adjustment of the annealing temperature to 54°C. This higher annealing temperature increased the

specificity of the reaction eliminating all the non-specific bands. PCR reactions using total RNA samples that were not reverse transcribed were also set-up as control to determine genomic contamination in the RNA samples.

All of the 20 µl PCR products, with the addition of 10 µl orange G loading dye (30% Ficoll, 2% Orange G, 0.5M EDTA, water 7.7% ddd water), were resolved in two percent agarose gels containing ethidium bromide. Four µl of a one Kb molecular weight marker (GIBCO-BRL, 10787-018) were run along with the samples. The images were viewed and analyzed using the Molecular Analyst Software and Statistical Analysis Software (SAS) (release 8.01 TS Level 01 MQ) t-test to determine the significance of the observed difference between the relative volumes of control and treated.

3. Results and Discussion

Tables 4-1 to 4-4 summarize the results of the Differential Display reactions. The bands of interest are those that are present only in treated samples but not in the controls, or those found in the controls but not in the treated, and those that are clearly visible in the treated and very faint in the controls and vice versa. These bands represent transcripts that are induced in the presence of salt stress, completely down-regulated or suppressed by the presence of salt stress, those that are normally expressed but are up-regulated in the presence of salt stress, and those that are partially down-regulated by salt stress, respectively. Exactly the same banding patterns were obtained from running duplicate samples indicating that the amplifications were specific. The Colony-PCR analysis of the inserts from the transformants on LB-Tet plates showed all selected colonies to contain the insert with expected size except the 1A and F11. In the agarose gels all the amplified inserts appeared as single bands within the range of expected size except,

Table 4-1. Samples of jack pine and black spruce seedlings germinated in vitro that were run in gel 1 and the corresponding number of differential bands observed and cloned

Lane #	Sample Code (Pooled seedlings germinated in vitro)	Number of Differential Bands Observed/ (Cloned)
1	JP1C	0
2	JP1 T3	0
3	JP2C	0
4	JP2 T3	0
5	JP3C	0
6	JP3 T1	0
7	JP4C	0
8	JP4 T1	0
9	JP5C	0
10	JP5 T1	0
11	JP6C	0
12	JP6 T1	0
13	BS2C	0
14	BS2 T1	12
15	BS5C	0
16	BS5 T1	8 (1A, 1B, 1C)
17	BS6C	0
18	BS6 T1	7

Figure 4-1 is image of gel 1.

Table 4-2. Samples of jack pine embryogenic tissues and surrounding calli that were run in gel 2 and the corresponding number of differential bands observed and cloned

Lane number	Sample code / Identity	Number of Differential Bands Observed/(Cloned)
1	1C JP3B	9
2	1T1	5 (2A 2B 2C 2D 2E)
3	100C JP6C-8	4 (F1 F2)
4	100T3	7
5	129C JP4Da-8	12
6	129T2	2
7	130C JP3D-b	6 (F3)
8	130T3	18 (F14 F15 F16)
9	131C JP2Bb	4 (F4)
10	131T3	0

Figure 4-2 is image of gel 2

Table 4-3. Samples of jack pine and black spruce clones (embryogenic tissues and surrounding calli) that were run in gel 4a Lanes 1-11 and gel 4b Lanes 12-20 and the corresponding number of differential bands observed and cloned

Lane number	Sample code / Identity	Number of Differential Bands Observed/(Cloned)
1	134C JP2Bb	3
2	134T3 JP2Bb	15 (F25-F26 F27 F28 F29)
3	139C JP2Dc	2
4	139T3 JP2Dc	6 (5A 5B 5C 5D 5E 5F)
5	138C JP4Ea001	4
6	138T3 JP4Ea001	27 (F20 F21)
7	128C JP3Da-5C	5
8	128T3 JP3Da-5C	17 (F22 F23 F24)
9	103C JP4EB-3C	5
10	103T3 Jp4EB-3C	28 (F30 F31 F32)
11	107C JP4G-2	4 (F5)
12	107T3 JP4G-2	15 (F34 F35)
13	244C JP4F	11
14	244T3 JP4F	4 (6A 6B 6C 6D)
15	232C JP4D2-A	2
16	232T3 JP4D2-A	10
17	32C BS2C-4	11
18	32T1 BS2C-4	37 (F37)
19	3C JP6D	1 (F6)
20	3T3 JP6D	39 (F38 F39 F40)

Figures 4-4 and 4-5 are images of gel 4a and 4b, respectively.

Table 4-4. Samples of jack pine seedlings in soil that were run in gel 3 and the corresponding number of differential bands observed and cloned

Lane number	Sample code / Identity	Number of Differential Bands Observed/(Cloned)
1	CA#25(111) Pre Treatment Tissue	3 (F7)
2	CA#25(111) Post Treatment Tissue	3
3	CB#35(111) Pre Treatment Tissue	2 (3A 3B 3C 3D F8)
4	CB#35(111) Post Treatment Tissue	4
5	T1#46(111) Pre Treatment Tissue	5 (F9)
6	T1#46(111) Post Treatment Tissue	4 (4A 4B 4C 4D)
7	T2#55(111) Pre Treatment Tissue	11 (F10)
8	T2#55(111) Post Treatment Tissue	4
9	CB#7(565) Pre Treatment Tissue	5 (F11 F12)
10	CB#7(565) Post Treatment Tissue	2
11	T1#10(565) Pre Treatment Tissue	5 (F13)
12	T1#10(565) Post Treatment Tissue	14 (F17 F18 F19)

Figure 4-3 is image of gel 3.

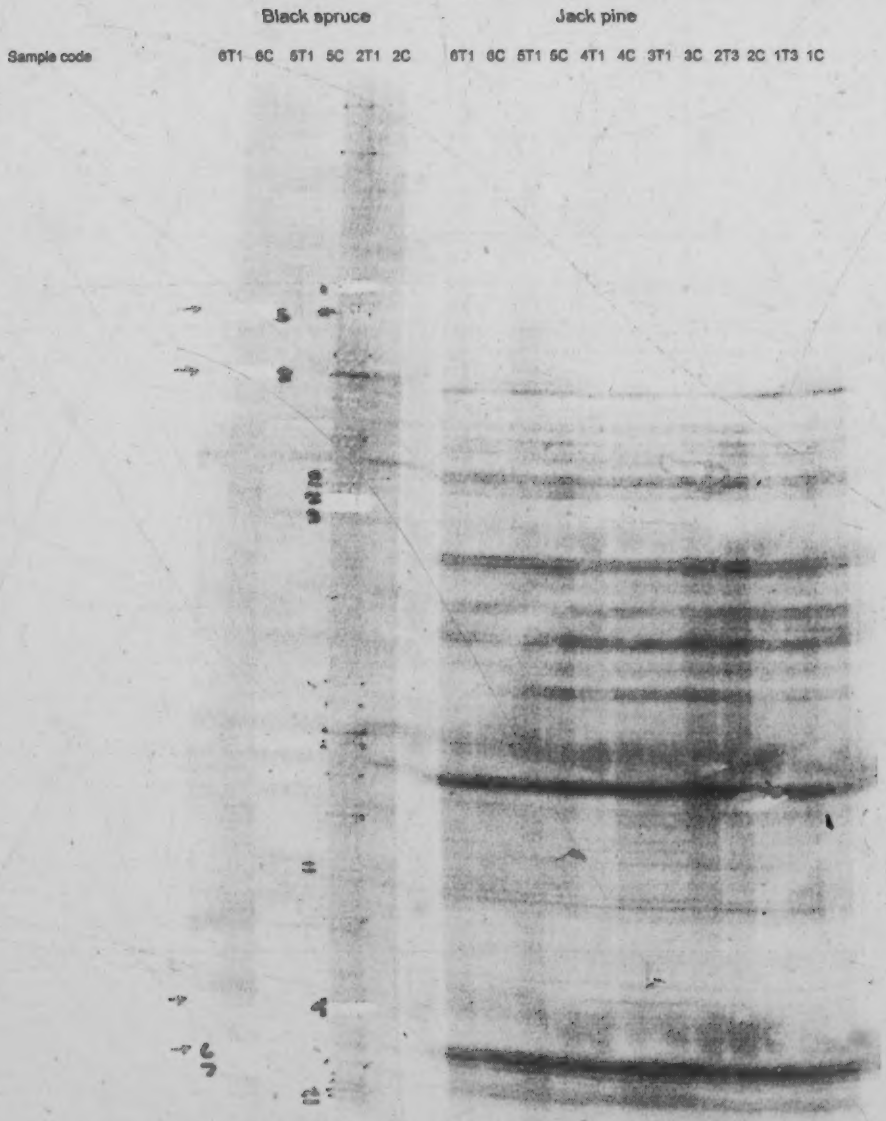

Figure 4-1. Autoradiogram of gel 1 showing differential fragments of jack pine and black spruce tissues from seedlings germinated and treated with sodium chloride *in vitro*. White spaces are locations of some differential bands that were cut from the gel.

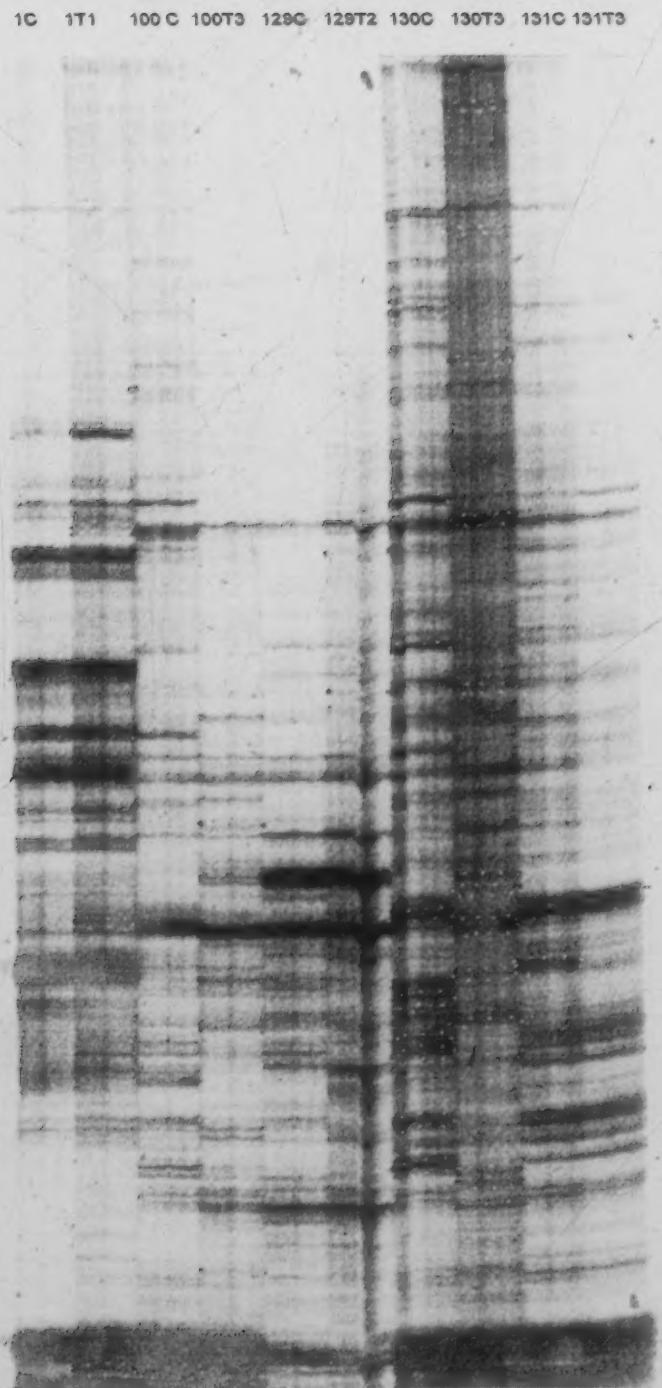

Figure 4-2. Autoradiogram of gel 2 showing differential bands from jack pine tissues developed through somatic embryogenesis and treated with sodium chloride *in vitro*.

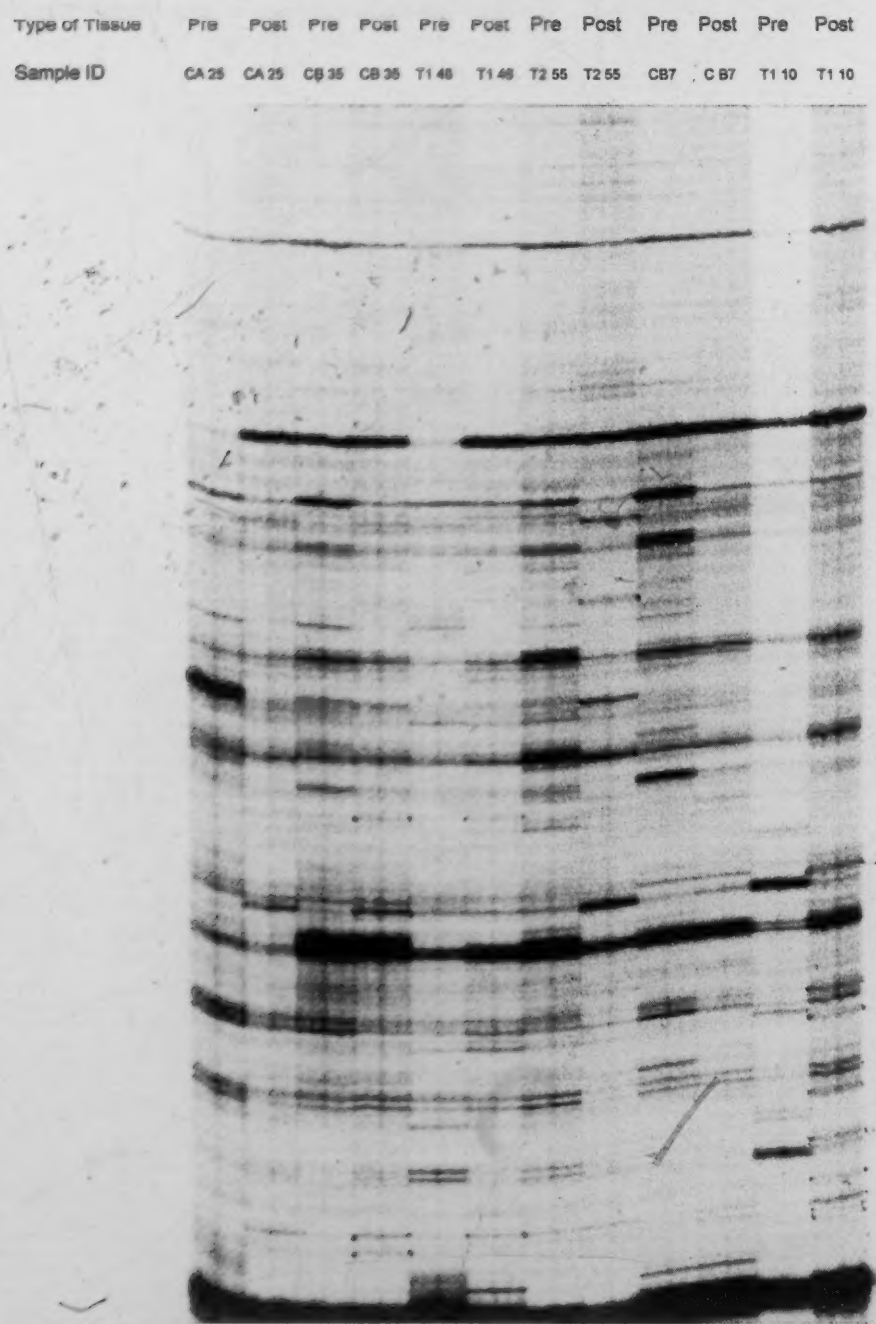

Figure 4-3. Autoradiogram of gel 3 showing differential fragments from shoots of seven-months-old jack pine seedlings grown and treated with sodium chloride in soil. Samples 1 to 8 belong to family 111, samples 9 to 12 belong to family 565 (Pre=pre treatment tissue; Post=post treatment tissue).

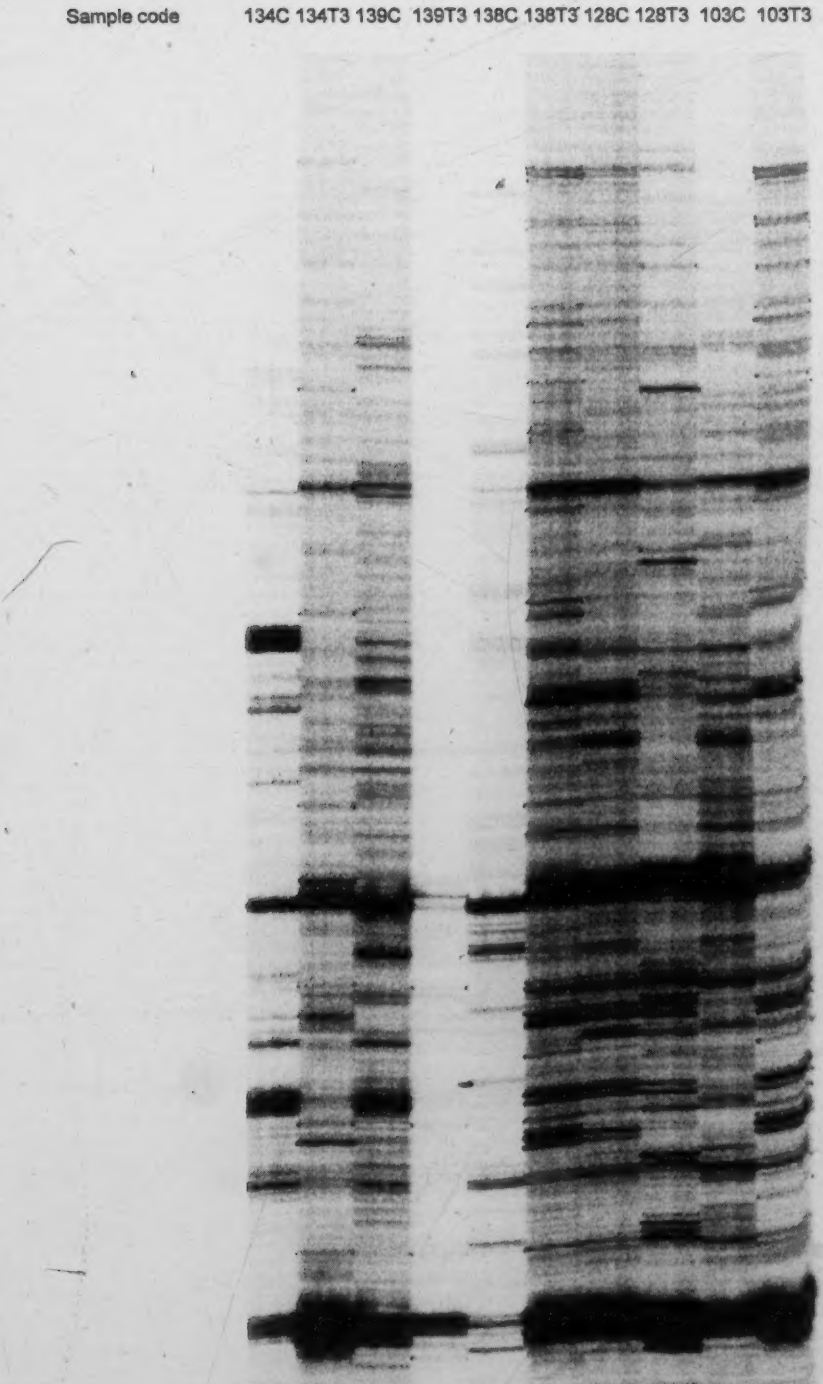

Figure 4-4. Autoradiogram of gel 4a showing differential bands from jack pine tissues developed through somatic embryogenesis and treated with sodium chloride *in vitro*.

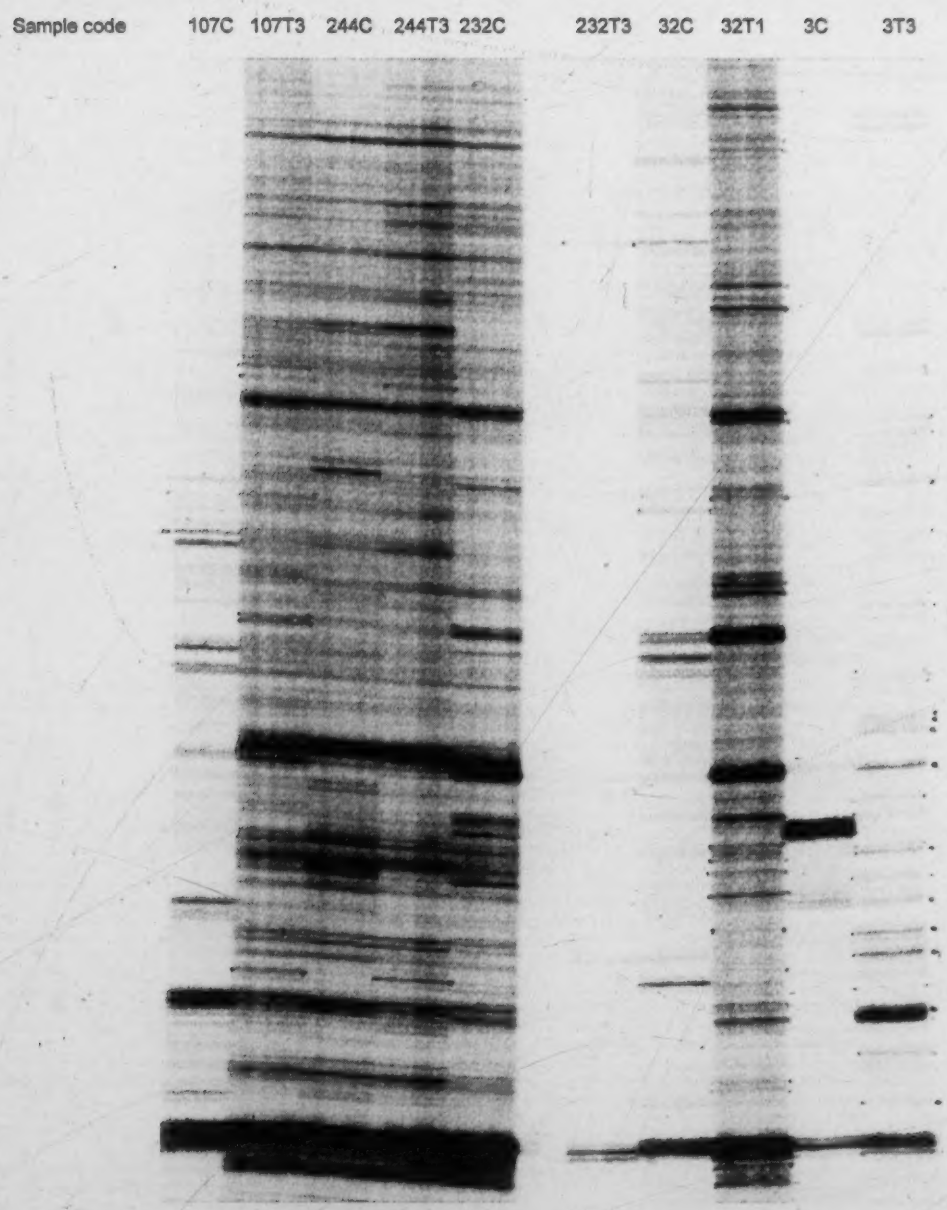

Figure 4-5. Autoradiogram of gel 4b showing differential fragments from jack pine and black spruce tissues developed through somatic embryogenesis and treated with sodium chloride *in vitro*.

1 A and F11 which were smeared, and in another run totally absent. This was consistent with the result of the sequencing, whereby Fragment 1A and F11 appeared not to have any inserts.

A total of 301 differential bands were observed from running 72 cDNA samples of control and treated tissue. Of the 66 selected bands, 64 were successfully cloned and sequenced. Eight were found to represent transcripts from down-regulated genes: F1, F2, F3, F4, F5, F6, F10, F13 and 47 were found to represent transcripts of up-regulated genes: 1B, 1C, 2A, 2B, 2D, 2E, 4A-4D, 5A to 5F, 6A to 6D F14 to F40. Fragments F7, F9, 3A, 3B, 3C, 3D, F8, F12 were isolated from pretreatment tissues of control plants. These bands were no longer visible in their corresponding post-treated tissues after six weeks. These fragments may represent genes that are possibly suppressed or down regulated as the plants advance in their development. These are not differential bands due to NaCl treatment, but were sequenced for the purpose of comparison with the other fragments. From the comparison, fragment 2C, which was isolated from a treated tissue, was found to have a 93% identity with Fragment 3A and therefore could not be considered under the group of up-regulated genes expressed in response to salt treatment. The sequences for all the fragments amplified using the Lseq and Rseq primers flanking the cloning site of the vector are found in Appendix 1.

The fragments were characterized by sequencing and by comparing their sequences with those reported in the SIB and GenBank databases. Table 4-5 and 4-6 summarize the results of the match found between these unknown fragments and the entries in GenBank and SIB. Thirty-eight of the fragments have significant sequence similarity to other fragments that were isolated. These are 1B, 1C, 2A, 2B; 2C, 3A; 2D, 3B; 3D, 4A, F4, F10; 4B, 5A; 4C, 6D; 4D, 5C; 5B, F23, F26; 5F, 6C; 6B. F22, F32; F7, F9; F12, F13; F28, F36; F33, F34; 5E, F2; F20, F21. Twenty-six of the fragments have no significant sequence similarity to any of the other cloned fragments: 2E, 3C, 5D, 6A, F1, F3, F5, F6, F8, F14, F15, F16, F17, F18, F19, F24. F25, F27, F29, F30, F31, F35, F37, F38, F39, F40. Twenty-three have sequence similarity with SIB entries: 4A, 3D, F4, F10, 5D, 5E, F2, 4B, 5A, F29, F33, F34, 2E, F20, F21, F28, F36, 5B, 5F, 6C, F23,

F26, F27. Fourteen of the fragments have sequence similarity to some of the entries in the GenBank: 1B, 1C, 2A, 2B, 4A, F4, F10, F16, F17, F24, F25, F29, F33, and F34. Thirty-three of the fragments: 2C, 2D, 3A, 3B, 3C, 4C, 4D, 5C, 6A, 6B, 6D, F1, F3, F5, F6, F7, F8, F9, F12, F13, F14, F15, F18, F19, F22, F30, F31, F32, F35, F37, F38, F39, F40, have no sequence similarity to any of the entries in the GenBank or SIB and may be transcripts of novel genes.

Table 4-5. Result of the search for sequence similarity between the cloned fragments and the GenBank entries

Fragment(s)	**GenBank entries that showed significant similarity (E=0.001 or higher)**
IB IC 2A 2B	*Pinus thunbergii* (Japanese black pine) chloroplast hypothetical protein 119 complete genome. L= 119707 ID: 1B=99/110(90%);ID IC= 99/109 (90%); ID 2A= 99/108(91%); ID 2B 98/108(90%) (E=1e-28, 7e-33, 4e-34, 6e-32)
4A F4 F10	*Pinus pinaster* partial mRNA for putative metallothionein-like protein L= 258 ID 57/67 (85%) (E= 8e-05, 1e-04, 3e-04)
F16	*Homo sapien* genomic DNA chromosome 8Q 23, (clone KB 1165G2) (E=1e-05)
F17	*Arabidopsis thaliana* genomic DNA chromosome 5 P1Clone: MTG10 L= 81284 ID 29/30 (96%)(E= 3e-04)
F24	*Pinus elliottii* PEC 30 mRNA L= 400 (E=5e-77) ID 201/217(92%)
F25	*Picea abies* mRNA for membrane intrinsic protein L= 1406(E=2e-24) ID 103/116(88%)
F29	Cloning vector lambda gt10 region flanking the cloning site L=1881 (E=8e-20) ID=60/63(95%)
F33 F34	Cloning vector lambda gt10 region flanking the cloning site L=1881(E (E=1e-37, 4e-38) ID F33 102/111 (91%); ID F34 82/82(100%)

Table 4-6. Result of the search for sequence similarity between the cloned fragments and the SIB entries

Fragment(s)	SIB entries that shows significant sequence similarity
4A	*Pinus taeda* NXSI_059_D08 5' mRNA sequence (Nsf Xylem side wood inclined) Length=303 ID=100/104 (96%) E=1e-42
3D F4 F10	*Pinus taeda* cDNA clone NXLV_045_C12 5', mRNA sequence (Nsf Xylem Late wood Vertical) Length=314 3D ID183/187=97% E=2e-89; F4 ID=159/162 =98% E=4e-79; F10 ID=237/253=93% E=e-100
5D	*Pinus taeda* cDNA clone PC09A05, mRNA sequence Pine TriplEx pollen cone library Length=380 ID=109/110 (99%) E=3e-53
5E F2	*Pinus taeda* cDNA clone PC01A07, mRNA sequence Pine TriplEx pollen cone library Length=291 5E ID= 37/38 (97%) E= 6e-08; F2 ID 38/39 (97%) E= e-10
4B 5A	664 Pt IPG2 *Pinus taeda* cDNA clone 9079M 3', mRNA sequence Length =351 4B and 5A ID 109/110(99%) E=2e-53
F29	EST0144 cucumber DD-RTPCR cDNA fragments *Cucumis sativus* cDNA clone CsSE25-1, mRNA length=109 ID=57/58 (98%) E=1e-22
F33 F34	EST0130 cucumber DD-RTPCR cDNA fragments *Cucumis sativus* cDNA clone CsSE10-3, mRNA sequence. Length=162 F33 ID= 29/29 (100%) E= 4e-07 F34 ID= 30/30 (100%) E=3e-08
2E	*Pinus Taeda* cDNA clone NXNV_095_E08 5' mRNA sequence NsF Xylem Normal Wood Vertical Length=253 ID 54/62 (87%) E=8e-12
F20 F21	*Pinus taeda* cDNA clone NXLV_032_D03 5', mRNA sequence. Nsf Xylem Late Wood Vertical) Length=457 F20 & F21ID 65/73(89%) E=1e-18
F28 F36	*Pinus taeda* cDNA clone ST72D12, mRNA sequence ST72D12 Pine TriplEx shoot tip library Length=467 F28 ID 91/105 (86%) E= 2e-18; F36 ID 98/102 (96%) E 2e-40
5B 5F 6C F23 F26 F27	*Pinus taeda* cDNA clone NXLV_033_C08 5', mRNA sequence Nsf Xylem Late wood Vertical Length=650 5B ID 105/105 (100%) E= 9e-53; 5F ID 171/172 (99%) E= 9e-91; 6C ID 170/172 (98%) E= 2e-88; F23 ID 124/125 (99%) E= 7e-63;F26 ID 118/118 (100%) E =2e-60; F27 ID 188/190 (98%) E= 1e-96

Fragments 1B and 1C isolated from pooled back spruce seedlings belonging to family five, and fragments 2A, 2B and 2Cisolated from jack pine family six have significant sequence similarity to a *Pinus thunbergii* chloroplast hypothetical protein gene. *Pinus thunbergii* is a known salt tolerant species. Up-regulation of transcripts that have functions related to photosynthesis may be some kind of a general response to salt stress among salt tolerant plants and was also observed in yeast (Yale &. Bohnert 2001) and in the salt tolerant cell lines of alfalfa (*Medicago sativa L.*) (Winicov and Krishnan 1996). Their study showed increased mRNA accumulation for both nuclear and chloroplast-encoded genes involved in photosynthesis. Koyro (1997), Yale &

Bohnert (2001) reported that salt stress triggers an active response requiring high-energy expenditure thus the need to increase photosynthesis and respiration in Sorghum plants (*Sorghum bicolor* x *S. sudanensis* cv. Sweet Sioux) and yeast (Saccharomyces cerevisiae).

Metallothioneins are small proteins that bind heavy metals and thereby protect cells against their toxic effects (King & Stanfield 1997). It is believed that similar proteins are working in the regulation of ions like Na^+ and Cl^- in plants. In this study, fragments F4, F10, and 4A, were found to have significant sequence similarity to a metallothionein-like protein gene of *Pinus pinaster*. These fragments were isolated from control tissue of JP2Bb clones and pre treat tissue of JP family 111, which means that the genes encoding them were down-regulated by salt stress at the pre-germination stage and at the seven-month stage of development. If these transcripts contribute to salt tolerance, then their functions may be related to binding salt ions and transporting them across the plasma membrane. Their suppression or down regulation is a rational mechanism whereby plants mitigate the influx of salt ions. This could be one of the mechanisms of tolerance to salt stress in jack pine. Fragment 4A, however, was identified from a treatment tissue of JP family 111 (seven-month-old seedling) and is an up-regulated transcript. Although F4, F10 and 4A have significant sequence similarity to metallothionein-like protein, but their sequences are not exactly the same as the others. Fragment 4A and F4 homology is 0.6, Fragment 4A and F10 is 0.02. They may be transcripts of different structural genes encoding for different but related metallothionein-like proteins. The responses of these different proteins to salt stress may vary depending on their function.

One of the fragments isolated, F16 has significant sequence similarity to a human DNA clone of unknown function. Trees are the most advanced organisms in the plant kingdom as human beings are to the animal kingdom. Despite their differences, some degree of similarity was found between them. This comparison needs to be further clarified.

Fragment F25, which was isolated from salt tolerant clone JP2Bb treated with 1.0% NaCl is significantly similar to a gene of an intrinsic membrane protein or porin of the salt-tolerant Norway spruce (*P.abies*). Major intrinsic proteins (MIP's) constitute a large protein family of ancient origin found in bacteria, fungi, animals, and plants (Johanson & Gustavsson 2002). The most recent studies investigating the nature and functions of porins provided strong evidence that F25 represent a gene for salt tolerance in jack pine. Intrinsic proteins act as channels to regulate and facilitate (Baiges *et al.* 2002) passive transport across biological membranes (Johanson & Gustavsson 2002, Hakman & Oliviusson 2002), such as the plasma membrane and the tonoplast (Hakman & Oliviusson 2002). Some of these intrinsic proteins seem to be able to transport other molecules such as glycerol or urea (Johanson & Gustavsson 2002, Baiges *et al.* 2002). A majority of these intrinsic membrane proteins are thought to be aquaporins (AQPs) that are specific for water transport (Johanson & Gustavsson 2002). It is therefore possible that F25 could be an aquaporin. Since aquaporins have been identified to be central components in plant water relations (Tyerman *et al.* 2002) and therefore in cell osmoregulations (Maurel *et al.* 2002), the expression of F25 could be a mechanism for tolerating salt stress in jack pine. Aquaporins are thought to be one of the mechanisms involved in plants maintaining optimal water balance in changing environmental and developmental conditions (Baegis *et al.* 2002). Exposure to different types of stress alters water relations and aquaporins may therefore be involved in stress responses (Baegis *et al.* 2002). Furthermore, the study of Baegis *et al.* (2002) has shown that transcriptional and/or post translational regulation of aquaporins would determine change in membrane water permeability. Their study also provided information on other roles possibly played by aquaporins, which include, among others, compatible solute distribution and micronutrient uptake. These roles have been previously mentioned to be important in maintaining homeostasis under salt stress conditions. Aquaporins are abundant, diverse, and expressed in large numbers in just a single plant (Baiges *et al.* 2002). This is possibly the explanation for the relatively high expression level of F25 found in the samples used in confirmation of its expression. F25 is one of the more interesting fragments isolated in this study. With the accumulating

evidence establishing its identity and function, it is believed to represent a good candidate gene for salt tolerance in forest trees.

Based on the results of the search for sequence similarity from SIB tabulated in Table 4-6, unknown fragments were matched to known genes to which they have sequence similarity (Appendix 2). Thirteen of the fragments, 4A, 3D, F4, F10, 4B, 5A, 2E, F20, F21, 5B, 5F, 6C, F23, F26, F27 have significant sequence similarity to *Pinus taeda* xylem wood mRNA. As mentioned in chapter two, one structural adaptation known in relation to salt stress is the sequestration of ions into large vacuolated cells of the older leaves and the osmolytes in the small-vacuolated meristematic cells. Another related mechanism is storage of Na^+ and Cl^- as abundant osmolytes such storage is however, limited by the available vacuolar space. Development of xylem wood provides additional vacuolar space. The expression of genes possibly related to the xylem wood seems to be a rational response to salt stress if one has to consider the fact that these types of tissue have more vacuoles that can serve important storage functions. Thick walled xylem wood cells that eventually die in can serve in ion storage. Storage of chloride ions in less sensitive areas of the plant such as vacuoles of ray cells and in the lumen and cell walls of tracheids has also been shown to occur in trees (Foster and Sands 1977). The expression of these transcripts related to xylem wood may be viewed as a strategy on the part of JP to develop sufficient vacuolar space for Na^+ and Cl^- storage.

Three fragments F29, F33, and F34 show homology to a cDNA clone of *Cucumis sativus*. If the function of this cDNA clone is related to development of succulent tissue, then the expression of F29, F33 and F34 may be viewed as another strategy related to salt tolerance, which is the dilution of ions through the development of succulent tissues. This mechanism has been known to exist in some halophytes, and possibly also in JP.

The sequences of the fragments that were isolated were compared with the sequences of some known salt tolerance genes such as CBF, CAN, DBF2, HAL 1, TPS1, DREB 1, CPK, SOS

1,2,3, using Bioedit, the Bionavigator and the Gene Jockey II program. None of the fragments from this study have significant sequence similarity to any of the known salt tolerance genes. This result seems to imply that the mechanisms working for salt tolerance in other plants exist in jack pine and black spruce, but the genes involved in these mechanisms may be different.

The results of this study suggest that the following mechanisms involved in salt stress response are working in JP and BS:

1) increase in energy production;
2) down regulation or up-regulation of certain genes involved in ion fixation or sequestration;
3) over expression of intrinsic membrane proteins that regulate ion influx, water relations, and selective absorption of nutrients for normal metabolism;
4) production of succulent tissue for ion storage; production of xylem vessels for transportation of ions into sinks, or for storage.

Ten of the longest fragments with significant sequence similarity to GenBank and SIB entries, F3, F10, 1C, 5D, F24, F25, F27, F28, F36 and F 39, were selected for confirmation of expression. Expression was confirmed through RT-PCR after Northern Blot failed to give any appreciable signal for several of these fragments. Gene specific primers (GSP) were designed using the Yahoo Primer3 software. Their sequences and annotations were tabulated on Table 4-7. Using these GSP, amplification products of expected size were successfully generated from reverse transcribed total RNA samples. The bands in the agarose gels were analyzed using the Molecular Analyst program. The expression of the ten fragments was confirmed by comparing the relative adjusted volumes (minus local background) of the bands from the control and the treated samples. The significance of the difference in expression between control and treated were determined by conducting a T-test on the adjusted volumes of the samples. Appendix 3 summarizes the results of the analysis using the Molecular Analyst program and the T-test using the SAS program.

Table 4-7. Sequences and properties of gene specific primers (GSPs) designed through the Yahoo Primer3 program

Primer	Source ID	Sequence	Melting temperature/ (°C)	G+C content
F3-R	JP3D-b	TGT CAG TTT AAG TCA AGT TCC AGT C	58.93	40.0
F3-L	JP3D-b	AAG CTT TGG TCA GTA GAC AGT GC	60.0	47.8
F10-R	JP111T2	CCC GTA ACC TGT AAT ATG AAG TCC	60.03	45.83
F10-L	JP111T2	GGC CAT AGA GGC TAC TCT ACC TTC	60.94	54.17
1C-R	BSF5T1	CTC GAT GAG TTC AAC AAT GAG TTC	60.17	41.67
IC-L	BSF5T1	CTT TGG TCA GGA TTC ATG TTC TTC	61.20	41.67
5D-R	JP2D-10T3	CCC TTC TAT TTC AAC AAA GAG C	57.22	40.9
5D-L	JP2D-10T3	GGT CAG AGA AGT CAA ATT ATG G	55.48	40.9
6D-R	JP4FT3	CCC CCT CAA ATA AAT CTC AA	56.63	40.0
6D-L	JP4FT3	CTT TGG TCA GCA AGG TTT TCT	58.50	42.9
F17R	JP565 PT T1	CGT TGC TAG TTT ATA TGG TG	51.15	40.0
F17-L	JP565 PT T1	TGG TCA GGA GAA GAA GAA TA	52.39	40.0
F24-R	JP3Da-5C T3	CAA AGG TGA TCC GCT TTA TTT C	59.97	40.91
F24-L	JP3Da-5C T3	AGC TTT GGT CAG CAC AGA GTC TAC	61.35	50.0
F25-R	JP2Bb T3	AGA GGC AAA GAG GTT GAG GTA ATC	61.29	45.83
F25-L	JP2Bb T3	TGT ACG GTG CAT ATT TCT GAG TTC	60.42	41.67
F27-R	JP2BbT3	AGC ATG CCG AAG TTA CAA TAA CAG	61.67	41.67
F27-L	JP2BbT3	GTG TTT AGG GGT ATG TTA GCC AAG	60.18	45.83
F28-R	JP2BbT3	CTT TAC AAG GAA AGG GAA CAT GAG	60.37	41.67
F28-L	JP2BbT3	GCA CCT ACC TAC ATT TTT CTT TGG	60.27	41.67
F36-R	BS2C-4 T1	AGA TAA GAT TGT GAA GGG GCT TTG	61.91	41.7
F36-L	BS2C-4 T1	CGG TAA ACA CAT CTA CAA TCA ACG	60.67	41.7
F39-R	JP6D-3 T3	GGT GTG TGT ACC AAG ACT AAT TGC	59.87	45.8
F39 L	JP6D-3 T3	TAC CTC GAA TTT AGG TCT CTC TCG	60.26	45.8

One constraint in the confirmation of expression of the fragments isolated in this study is having multiple replicates of a single pair of samples of total RNA. To address this problem, the same set of total RNA samples was tested to confirm expression of all the ten fragments. In that way a difference in expression can be readily observed when one sees the differences in the volume of the samples amplified by the different GSPs. The specific details for each of the samples and each of the fragments confirmed are tabulated in Appendix 2. The results of the RT-PCR confirm the existence of these fragments in the population of JP and BS spruce included in this study. There is, however, considerable variability among samples used to test expression as the RNAs were extracted from different individuals. Thus, in spite of the big difference in the

adjusted volume between control and treated (per sample pair) and in the mean between pooled control and pooled treated, the T-tests generally resulted to an insignificant difference. At alpha= 0.05, only Fragment 25 showed a significant t-test result with Pr value of 0.0207. This result seems to imply that F25 is a common gene expressed in response to salt stress because in spite of the variability among the samples it is consistently highly up-regulated. The other fragments seem to be expressed quite uniquely in every sample examined.

It is known that salt tolerance expressed in response to salinity stress is a multigenic trait (Foolad 1997, Hoberg 1998, Flower *et al.*1997, Winnicov 1998, Zhong & Dvorak 1995). The result of this study seems to indicate that JP or BS families or individuals which are most tolerant to saline stress are the ones having the greatest number of these "salt tolerance genes", all fully expressed. At one extreme would be those families or individuals which have none of these genes, and are therefore the most sensitive. Those with a few genes that maybe are blocked due to gene interaction may be more or less sensitive. The variation in sensitivity of plants to salt stress may be related mainly to the number of salt tolerant genes possessed and expressed and whether or not these genes are normally constitutive or require induction. Although the number of samples tested may not be sufficient to make a conclusive statement supporting this idea, the results do suggest, there is reason to believe that this seem to be how genes for salt tolerance are working in jack pine and black spruce. Family two which seem to contain all the genes that had been discovered had the most number of surviving individuals following the screening stage. The same is true with family six. This observation may explain the increase in vigor relative to salt stress as plants are gradually exposed to the stress. As the plant is gradually exposed to the saline stress, more genes are stimulated, consequently there would be an observed increase in vigor. Furthermore, it may also explain why plants at the younger stage of development tend to be more sensitive to salt stress than when matured. It was reported in several studies that as plants mature their resistance or tolerance to salt stress increases. As the plant is exposed to increasing environmental stress, more genes are elicited and became expressed. Generally, in this study the pre-germination tissues, embryogenic tissues and their surrounding calli, showed

more survivors to 1.0% NaCl than the seven-month-old seedlings treated with the same salt concentration. It is possible that part of plant's strategy for survival, is that genes needed for defense are active within a seed prior to germination. As the seed germinates, in the absence of stress, some of the genes are not necessary and are shut down. JP and BS as glycophytes, require time to reactivate these genes.

To date there is not enough information available to explain the mechanisms by which genes related to salt tolerance are expressed in trees, and nothing was available for pine and spruce. Thus, it is difficult to make further speculation on the results of this study. Further study of the fragments isolated should result in additional information that may answer some of the questions about salt stress responses in trees that presently remain unanswered.

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Chapter V

Summary, Conclusions, and Recommendations

1. Summary

Salt stress is an environmental problem affecting mainly plant productivity and water quality. The impact of salinity on forestry is extensive and costly, (Allen *et al.*1994) affecting wetlands, mining lands, forest adjacent to mining sites (Rincon 1980, Auchmoody & Walters 1988) and roadside trees (Dochinger & Townsend 1979). Reforestation and revegetation could be done efficiently only with the use of carefully selected salt tolerant plants. The current practice in screening for salt tolerance is by growing seedlings in high salinity solutions. This is costly due to the need in screening large number of seedlings in order to find a few salt-tolerant trees. A plausible alternative is to select for genes that confer salt tolerance in tree species. These genes will be valuable for selecting native trees to become a source of salt tolerant trees for propagation. Planting seeds from these select trees on salt-stressed environment would be cost effective and environmentally responsible.

Using Differential Display RT-PCR, transcripts related to salt stress response can be identified and isolated and later characterized by cloning, sequencing and comparing their sequences to known genes and expressed sequence tags in the databases. These transcripts derived from the analysis of total RNA of plants exposed and unexposed to saline stress could lead to the identification of genes encoding proteins that impart salt tolerance, thereby setting the foundation for improvement. This study aimed to lay the groundwork for understanding the genetic basis of salt stress response in jack pine and black spruce.

Part 1 of this study focused on screening for salt stress responses to obtain suitable materials for DD RT-PCR. The screening was done at three stages of plant development

(pre-germination, germination, and at five-and-a-half-months-old) using different concentrations of sodium chloride (0.00 mM, 85.51 mM, 119.72 mM, 171.03 mM, 256.54 mM). At the pre germination stage, embryogenic tissues derived from zygotic embryos were used as explants for every cell line. A total of 600 zygotic embryos from six families of jack pine and 600 zygotic embryos from six families of black spruce were cultured through somatic embryogenesis using modified 1/2Litvay medium containing 0 mM, 85.51 mM, 119.72 mM, 171.03 mM NaCl. One hundred seeds from each of the six families of jack pine and 100 seeds from each of the six families of black spruce were germinated in modified ½ MS medium containing 0 mM, 85.51 mM, 119.72 mM, 171.03 mM NaCl. Seventy-four four-and-a- half-month old seedlings of jack pine in soil from Family 111 and 565 were divided into treatment groups. The seedlings were watered with 0 mM, 85.51 mM, 119.72 mM, 171.03 mM, 256.54 mM NaCl solutions twice a week. All treatments were continued for 6 weeks. At the end of 6 weeks "Control" and "Treatment" tissues were harvested and frozen at –80°C until needed. Diagram 3-1 summarizes the screening procedure.

Part II of this study was the identification and isolation of transcripts resulting from differentially expressed genes. Total RNA was extracted from 72 samples of "Control" and "Treatment" tissues at different stages of development using the modified Yinghua Huang Protocol. Using the GenHunter RNA Image™ Kit, these total RNA samples were reverse-transcribed into cDNAs, amplified through PCR, and resolved in PAGE gels using infra-red labeled dATP and later α-[^{33}P]dATP. The bands of interest were those that were present only in treated but not in control, those found in the control but not in the treated, and those that were clearly visible in the treated but very faint in the control, and vice versa. These represent transcripts that are induced in the presence of salt stress, completely down-regulated or suppressed by the presence of salt stress, those that are normally expressed but are up-regulated in the presence of salt stress, and those that are partially down-regulated by salt stress, respectively.

After getting exactly the same banding patterns from duplicate samples indicating that the amplifications were specific, bands of interest were excised from the gels, purified, reamplified, cloned and sequenced. The sequences were compared among themselves and with the entries in the GenBank and Swiss Institute of Bioinformatics (SIB), and to sequences of known salt-tolerance genes using the Gene Jockey II, Bioedit, and Bionavigator programs.

Differential display generated 301 differential bands from 72 samples. Of these, 66 fragments were selected for cloning and sequencing. Of these 66 fragments, eight represent transcripts of down-regulated genes, 47 represent transcripts of up-regulated genes. Two of the fragments were not successfully cloned. Thirty-eight of the fragments showed significant sequence similarity with the other cloned fragments (i.e. 1B, 1C, 2A, 2B) and 26 have no significant sequence similarity with any of the other cloned fragments (i.e. F24, F25, F27). Twenty-three have significant sequence similarity with entries in the Swiss Institute of Bioinformatics (SIB) (i.e. 4A, 3D, F4, F10), and 14 have significant sequence similarity with entries in the GenBank (i.e. F17, F33, F34). Thirty-three of the fragments (i.e. 2C, 2D, F22) showed no significant sequence similarity with any of the SIB and GenBank entries and may represent transcripts of novel genes. GenBank and SIB were searched for sequence similarity. Four of the fragments were found to have significant similarity to *Pinus thunbergii* chloroplast hypothetical protein, one of the fragments to human DNA sequences, three of the fragments to *Pinus pinaster* partial mRNA for putative metallothionein-like protein, one of the fragments to *Arabidopsis thaliana* chromosome 3, one of the fragments to *Pinus elliottii* PEC mRNA, one of the fragments to *Picea abies* mRNA for membrane intrinsic protein, 13 of the fragments to different types of *Pinus taeda* Xylem and clone mRNA, two of the fragments to *Cucumis sativus* mRNA.

Ten of the longest fragments with significant sequence similarity to GenBank and SIB entries, F3, F10, 1C, 5D, F24, F25, F27, F28, F36 and F39, were selected for confirmation of

expression using the RT-PCR technique. Using the Yahoo Primer3 program, gene specific primers (GSPs) were designed based on the sequences of these 10 fragments. Using reverse transcribed total RNA from 10 pairs of samples, amplification products with expected size were successfully generated using these GSPs. The results were analyzed using the Molecular Analyst and SAS program.

The results of this study have shown that:

1) There is considerable intraspecific variability within jack pine and black spruce in response to salt stress. This variability was observed among individuals within families and among families within the species.
2) Jack pine are more responsive to induction during somatic embryogenesis than black spruce. Of the 600 cell lines that were induced, 415 (69.17%) were jack pine and only 73 (12.17%) were black spruce. Regeneration of JP and BS using somatic embryogenesis is poor, only nine of the 600 cell lines (1.5%) were able to develop mature somatic embryos. Jack pine had a lower regeneration percentage than black spruce: jack pine 2/300 (0.66 %) and black spruce 7/300 (2.33%). The protocol used for mature jack pine seeds was actually modified from the protocol optimized for spruce and larch immature seeds. A much higher percentage of regeneration might be obtained if the protocol were further optimized for use with mature jack pine seeds. Although the percentage of cell lines that reached maturation is relatively low, the number of mature embryos per cell line is very high, about 100 or more mature embryos in less than one gram of embryogenic tissue.
3) Most jack pine and black spruce plants can tolerate 0.5% NaCl. At the callus stage (pre-germination stage), there were more JP cell lines that could tolerate 1.0% NaCl than BS cell lines. Embryogenic calli showed a higher tolerance to salt than seven-months-old seedlings. There were 27.87% (68/244) JP embryogenic cell lines showing at least 1% growth (increase in weight after 6 weeks of salt treatment) in 1.0%-NaCl concentration

while only 6.67% (1/15) of the seven-month-old seedlings that retained at least 85-100% green tissue under the same NaCl concentration. Both JP and BS showed very high sensitivity to sodium chloride stress at the germination stage to about six-weeks-old. At the same stage of development, black spruce is more sensitive than JP. Sixteen percent (96/600) of JP explants survived the 0.5% NaCl treatment for six weeks, compared to only three percent (18/600) of BS explants. BS also showed an earlier arrest of the development in the selective media. The results of this study support the findings of other studies that seeds of many plants have a relatively higher tolerance to environmental stress than their very young seedlings and that tolerance can develop with age (Shannon *et al.* 1984). Several studies have shown that salt tolerance is a multigenic trait (Foolad 1997, Hoberg 1998, Flower *et al.* 1997, Winnicov 1998, Zhong & Dvorak 1995) and that different genes control salt tolerance at different stages of plant development (Mano & Kayzuyoshi 1997, Foolad 1996). The transcripts related to salt stress response that were isolated in this study were many and varied, it is probable that multiple genes control the response of JP and BS to salt stress. The observed difference in sensitivity among clones derived from somatic embryogenesis, newly germinated seeds, and seven month-old seedlings implies that different genes responsible for the salt stress responses may be working at different stage of development in JP and BS. The mechanisms for salt tolerance that maybe at work in JP and BS appear to be the same as the mechanisms at work in other organisms. These mechanisms may include increases in energy production, down regulation or up regulation of certain genes involved in ion fixation or sequestration, over expression of intrinsic membrane proteins that regulate ion influx and water relations, and are involved in selective absorption of nutrients for normal metabolism, structural adaptation through the production of succulent tissue for ion storage; production of more xylem vessels for transportation of ions into sinks, or for storage, and the transport of ions in older leaves which eventually senesce.

4) Although the mechanisms of response to salt stress in JP and BS appear similar to those at work in other organisms, the results of this study suggest that at least some of the

genes that control these mechanisms in trees are different. The comparison between these different transcripts and known genes for salt tolerance such as CBF, CAN, DBF2, HAL1, TPS1, DREB1, CPK, SOS 1, 2, 3 did not show any significant similarities.

2. Conclusions

(1) Transcripts related to salt stress response that were identified and isolated from jack pine and black spruce are many and varied, implying that the response in JP and BS are governed by multiple genes.

(2) The mechanisms that are involved in salt stress response in JP and BS may include an increase in energy production, down regulation or up regulation of certain genes involved in ion fixation or sequestration, over expression of intrinsic membrane proteins that regulate ion influx and water relations, and are involved in selective absorption of nutrients for normal metabolism, structural adaptation through the production of succulent tissue for ion storage; production of more xylem vessels for transportation of ions into sinks, or for storage, and the transport of ions in older leaves which eventually senesce.

(3) It is possible that different sets of genes controlling the mechanisms in salt stress response are found in JP and BS.

3. Recommendations

Results suggest the complete gene(s) expressing these transcripts should be isolated and characterized, and their contribution to salt tolerance assessed and confirmed. A long list of responsive genes has been produced by molecular studies, however it is now widely recognized that many salt responsive genes do not contribute to tolerance; rather, their induction reflects salts stress damage (Zhu 2000). Furthermore Zhu (2000) reported that molecular approach has mostly identified genes or gene products based only in their expression, but many genes that are important for salt tolerance may not be induced by salt

stress. In light of these findings, it is recommended that transcripts that were isolated from this study be further evaluated for their contribution to salt tolerance. Genes which expressed these transcripts have to be isolated and characterized. It is further recommended that other fragments whose expressions were not confirmed should be validated using samples that were screened for salt tolerance and are currently kept in the -80°C-freezer at the Molecular Biology and Biotechnology Center, Agro-Forestry Building, University of Alberta, and from the natural populations.

The reliability of the selection of the differentially expressed fragments is mainly based on the skill of the researcher to carefully isolate the bands of interest without including the background. In this study it is assumed the fragments that were cloned and sequenced are truly the differential bands. However, considering the limitation of the technique to identify true differential bands that was used in this study, the researcher recognizes the need to further analyze the fragments that were discovered to provide more evidence that these are truly differential fragments.

Somatic embryogenesis is a very promising technique in generating mature embryos from mature jack pine and black spruce seed. It is therefore recommended that the protocol used in this study should be further optimized to produce jack pine and black spruce embryos for research and other purposes.

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CHAPTER VI

APPENDIX

Appendix 1. Sequences of fragments amplified using Lseq and Rseq primers flanking the cloning site of the vector and the corresponding GenBank accession numbers for those that were submitted to dbEST as expressed sequence tags.

1 B (CA844429)

AAGCTTTTTTTTTTTAATAA GATCTCGATGAGTTCAACAATGAGTTCTATATTACAATATAGAA
TCCATTATTTTTTTTTACTATATAAGTAAAATAGTGCGAAGAACATGAATCCTGACCAAAGCTT

1C (CA844430)

AAGCTTTGGTCAGGATTCATGTTCTTCCACTATTTTACTTATATAGTAAAAAAAAATAATGGAT
CTCTATATTGTAATATAGAACTCATTGTTGAACTCATCGAGATCTTATTAAAAAAAAAAAGCTT

2 A (CA844389)

AAGCTTTGGTCAGGATTCATGTTCTTCCACTATTTTACTTATATAGTAAAAAAATAATGGATCT
CTATATTGTAATATAGAACTCATTGTTGAACTCATCGAGATCTTATTAAAAAAAAAAAGCTT

2 B (CA844390)

CTTTGGTCAGGATTCATGTTCTTCCACTATTTTACTTATATAGTAAAAAAAAATAATGGATCTC
TATATTGTAATATAGAACTCATTGTTGAACTCATCGAGATCTTATAAAAAAAAAAAAGCTT

2C

AAGCTTTGGTCAGTGATCCCGAATGGAGAATGCATCCACCCATTGTATTCATGCACCTATGG
AGTTTACCCATGGTATTGTAAGTGGCATCCATCCATGGTATTCATCCTCTCATGGAATTTACC
CATGGTATTGTAAGTGGCATCCACCCTTGGTATTCATCCTCCAGTGGTATTTATCCACTCATG
GAATTTATGCATAGCATTGTAAGTGACATCCACCCATGTATTCATTCACCTATGGTATTTACC
CACCCATTGAATTT

2 D (CA844391)

AAGCTTTGGTCAGAGGCGGACAGGAAACATGGGAACCAATTCCTTAACTTATAGAGTTGTTA
TGTTTTACCGCCTATTGTTAAATCAATATGTATGTATCCACAAACTTGGGAAAATCTCTGAAAT
TGATCACATAGTTGCAACTAAGAAAAGATGTGAACTAATCATAAATGTCACGGTCTTGCAAGG
CTGTTCGGCACTTATCATTTTAGACTCACCCTTTGTAATCCGTTCTTTGGCATTTAGGCCAAG
TTTCTAATATATC

2E (CA844392)

AAGCTTTGGTCAGGTATGAGTCCTGTCCTGGTGAACTGATAAATGTTTTGAATTTCTGAATCA
GTTTTGTATATAATTTTTCATAATTGTAAAGAATAATATACAGTGGAGAATCAAGGTCAAGTTC
AGGGGTATTTGATAGGCCTATGGTATGGTATCGTGCACTTTTTACACTGGACATAAAGTGTTA
TTTGATAGGCTTATGGTATGCTACCATCCACCTTTTTCATTAGGCACAATCAGATACAACTGG
CTTCCAGAC

3A

CTTTTTTTTTTTACAATAGCACAGGTAAATTCAATGGGTGGGTAAATACCATAGGTGAATGAA
TACATGGGTGGATGTCACTTACAATGCTATGCATAAATTCCATGAGTGGATAAATACCACTGG
AGGATGAATACCAAGGGTGGATGCCACTTACAATACCATGGGTAAATTCCATGAGAGGATGA
ATACCATGGATGGATGCCACTTACAATACCATGGGTAAACTCCATAGGTGCATGAATACAAT
GGGTGGATGCATTCTCCATTCGGATCACTGACCAAAGCTT

3B

AAGCTTTGGTCAGAGGCGGACAGGAAACATGGGAACCAATTCCTTAACTTATAGAGTTGTTA
TGTTTTACCGCCTATTGTTAAATCAATATGTATGTATCCACAAACTTGGGAAAATCTCTGAAAT
TGACACATAGTTGCAACTAAGAAAAGATGTGAACTAATCATAAATGTCACGGTCTTGCAAGG
CTGTTCGGCACTTATCATTTTAGACTCACCCTTTGTAATCCGTTCTTTGGCATTTAGGGCAAG
TTTCTAATATATCCACTCCCCGTTTCTGTAAAAAAAAAAAGCTT

3C

AAGCTTTGGTCAGACAATATAGCCGTTCCCTCTTTAATTTCAATTACTATAGAGGCAGAAATG
AGGAATGTGGGTCTGCGTACCCGGTTTTGAACCTCTTCGCCCAAACTTTCGATAGCTATGTA
ATAGAACTTCGTTTCTGTTTTTTATAATACAAAAGTACCCGACTTCGTTAAAAAAAAAAAGCTT

3D

TTGGTCAGACCCAATGGATGGTTGTGCAGGGCCATGGAGGCTCCTCTACCTGCACTCTTTAA
ATAAATAAAGACTTCAGTTTAGGTAGTCTTGGGACTTTCAGTATGGCTTGTACCGTTAATGGC
CTGTTAATTATTATGCTGTGTCTCTGCAGCTTAAGCTATTTCTGACTTCGTCTTTAAATGCTAA
AAAAAAAAAGCTT

4A (CA844431)

AAGCTTTTTTTTTTTACTGAAAGTCCCAAGACTACCTAAACCGAAGTCTTGATTTATTTACGAG
TGAAGGTAGAGTAGCCTCTATGGCCCTGCACAACCATCTATTGGGTCTGACCAAAGCTT

4B (CA844432)

AAGCTTTGGTCAGCTAATAAAAACATCCATATCATTATATGGAGGAGGTGGTGGAGTTGATTC
GGAGCATAGAAGATACCATATAACATTATAAAATATGTGGCTTCATCTTATTATAAAAAAAAAA
AGCTT

4C (CA844433)

AAGCTTTTTTTTTTTACCCCCTCAAATAAATCTCAAATGAGATTGAATATATTAGCAAATTGTT
GATCATAATTGGATAGTGGCTTAATATAGTAGAATACATATTATCATTTCCAGAAAACCTTGCT
GACCAAAGCTT

4D (CA844434)

AAGCTTTGGTCAGTAGTAAATCTTTCAAGCAACACCTCTATGCTTATTTCCTTGGTATTTTAAT
AGAAGTGGTCATTGAAGTGCTATGAGCACCATCTCGATGCTTATGCTTATTTTGTTGACATTT
TAATAAATGTTGTTTTGAGAAGAATTGACTTTATCAATGATAGATATTTGACAACTCCTTTCTA
TATAAAAAAAAAAAGCTT

5A (CA844393)

AAGCTTTGGTCAGCTAATAAAAACATCCATATCATTATATGGAGGAGGTGGTGGAGTTGATTC
GGAGCATAGAAGATACCATATAACATTATAAAATATGTGGCTTCATCTTATTATAAAAAAAAAA
AGCTT

5B (CA844394)

AAGCTTTTTTTTTTTAGTAAAATAATAATTAAATTTCCCTTTTACTCCAAAATGCCCGAAATTAA
AGCCACTTGGCTAACATACCCCTAAACACAAACAAAAATGTAATGTCCTGACCAAAGCTTC

5C (CA844395)

AAGCTTTGGTCAGTAGTAAATCTTTCAAGCAACACCTCTATGCTTATTTCCTTGGTATTTTAAT
AGAAGTGGTCATTGAAGTGCTATGAGCACCATCTCGATGCTTATGCTTATTTTGTTGACATTT
TAATAAATGTTGTTTTGAGAAGAATTGACTTTATCAATGATAGATATTTGACAACTCCTTTCTA
TATAAAAAAAAAAAGCTT

5D (CA844396)

AAGCTTTGGTCAGAGAAGTCAAATTATGGCAGAATCTAATCCGTCGGTTACCTTGTCCATGA
AAGGGAGTATATATCTTTAAACGAGCGACATTAAAAACTTACAATGCTCTTTGTTGAAATAGA
AGGGTTTTCTTACTAAAAAAAAAAAGCTT

5E (CA844397)

AAGCTTTTTTTTTTTTAGAAATCAAAATATTCCATTTTCCAAATTACCCAAAATATTGACGATCAG
TTTCTCTATGGACATGACAAGACCGCCATATACTCAAAATCTCTAAAATTATTCA ATGMTG AA
AGAACCAAATTTATATCATTCTGCTCTTTCCCTGACCAAAGCTT

5F (CA844398)

AAGCTTTGGTCAGGACATTACATTTTTGTTTGTGTTTAGGGGTATGTTAGCCAAGTGGCTTTA
ATTTCGGGCATTTTGGAGTAAAAGGGAAATTTAATTATTATTTTACTATTCTGTTATTGTAACTT
CGGCATGCTTCTCTTTCTTTATTCAAGTAATCAGAATTGAGTAGATACTAAAAAAAAAAAGCTT

6A (CA844399)

AAGCTTTGGTCAGGGAAAGACACCGGAGAAGAMAATAAAGCCAGCGGCAGCAGATGCAAG
CCTTGAACAACATGCTGAAGAACAGAAGACTACTAAAAAAAAAAAGCTT

6B (CA844400)

AAGCTTTGGTCAGCATTGCCTTGCCTTTCTCTATATAAAGTATAAATATAGTGGAATAAACTC
CGCCAACGCGTAGGCGATCAAGTAGGCAGAATGACTTTTGTCTTTGGAAATTGAAATAATAT
TAATGATGTTGTAAAAAAAAAAAGCTT

6C (CA844401)

AAGCTTTTTTTTTTTTAGTATCTACTCAATTCTGATTACTTGAATAAAGAAAGAGAAGCATGCCG
AAGTTACAATAACAGAATAGTAAAATAATAATTAAATTTCCCTTTTACTCCAAAATCCCCGAAA
TTAAAGCCACTTGGCTAACATACCCCTAAACACAAACAAAAATGTAATGTCCTGACCAAAGCT
T

6D (CA844402)

AAGCTTTGGTCAGCAAGGTTTTCTGGAAATGATAATATGTATTCTACTATATTAAGCCACTATC
CAATTATGATCAACAATTTGCTAATATATTCAATCTCATTTGAGATTTATTTGAGGGGGTAAAA
AAAAAAGCTT

F1 (CA844422)

GAAGCTTTGGTCAGGGGTTCTGATCTTGTACATATGTTCTATTTATTTGAATTCTCATCTACTG
GTAGATACCTTCGCAACTCTCCATCATGCCAGTGTTATGAGGATCTGCTCTTAAATCAAGAG
CTAAGAGCTAATATTCTTGCTGTAA AAAAAAAAAG CTT

F2 (CA844423)

GAAGCTTTGGTCAGGGAACGAGCAGAATGATATAATTTGGTTCTATTTCATCATTGACTAATT
TTAGAGAGTCGTAACATGTATATTTTGCCTTCTAATTCCACCATTTTCAACTTTATTATATCTCT
ACTGACTAATGGAAGAAATTCAATGTACAGAAATGACTTGTAGGACGTTTCTCTCATTTCATT
ATG TTCAACTGTACTGGTTCTCTAGTAAAAAAAAAAAGCT

F3 (CA844423)

AAGCTTTGGTCAGTAGACAGTGCCTTCGTCCATTGGTGATATGGAGTCATTATTTCGACAAA
CAACACAAGACTTTGACACATTTATTGACTGGAACTTGACTTAAACTGACATTAATTATGAAAC
CAACATGA CTAAAAAAAAAAAGCTT

F4

CTTTTTTTTTTTAGCTTAAGCTGCAGAGACACGGCATAACAATTAACAGGCCATTAACGGTA
CAAGCCATACTGAAAGTCCCAAGACTACCTAAACTGAAGTCTTTATTTTTACGAGTGCAGGTA
GAGGAGCCTCCATGGCCCTGCACAACCATCCATTGGGTCTGACCAAAGCTT

F5 (CA844425)

CTTTTTTTTTTTACAGGATAATAGAAACACTTCGCATTAAACTGAGTCAACAAGAAAGGTACTT
TTCTCCAATGTAGAAGCAATTGACAATGCCACCTGATTCTCAATCTGACCAGGTAGAGCCAT
ACCCCCAACACACAAACTCCCTGATCCTTGGGATCATATAAATTACATTTATCCACATCCTAG
TAGACATGGCTGACCAAAGCTT

F6 (CA844426)

AAGCTTTTTTTTTTTTAGAGGAGATTTAGGGAACAATTTTATAAGCATCTTAAAGCGTTGGATGT
TCTATCTGTATATATAAATCAAATGAAATCTCCAAGTCCTATCCAACAAGTGCAGTTAAAAACT
ACAAAGCATCAACCACACAAAATATCAATTTGCATCCTGACCAAAGCTT

F7

AAGCTTTTTTTTTTTTAGAAATCCACTTTTATAATCTGAAAACATATCACACATCATGCCATTGA
CTCCTGAAATCTCTCCATCCTCTCTTGCTAACCAGAAAATCCAAATCCATGTAAACAGAACCA
AGCCTAGGAATACAAAATGGTCTTTTGTACAGAACGTTTCCCAAGCCTACCAAGAAAACCAA
CATAACATTTAGTATAACAGCAGCAACTTGACTCAATACAGACAAAGAAATGCAGCAACCTGA
CCAAAGCTT

F8

AAGCTTTTTTTTTTTTAGTAGTCTTCTGTTCTTCAGCATGTTGTTCAAGGCTTGCATCTGCTGCC
GCTGGCTTTATTTTCTTCTCTGGTGTCTTTCCCTGMCCAAAGCTT

F9

AAGCTTTTTTTTTTTTAGAAATCCACTTTTATAATCTGAAAACATATCACACATCATGCCATTG
ACTCCTGAAATCTCTCCATCCTCTCTTGCTAACCAGAAAATCCAAATCCATGTAAACAGA
AACAGCCTAGGAATACAAAATGGTCTTTTGTACAGAACGTTTCCCAAGCCTACCAAGAAA
ACCAACATAACATTTAGTATAACAGCAGCAACTTGACTCAATACAGACATAGAAAAAAAG
CAACCTCCCCAAAGCTT

F10

AAGCTTTGGTCAGACCCAATAGATGGTTGTGCAGGGCCATAGAGGCTACTCTACCTTCACTC
GTAAATAAATCAAGACTTCGGTTTAGGTAGTCTTGGGACTTTCAGTATGGCTTGTACCGTTAA
TGGCATGTTAATTATTATGCTGTGTCTCTGCAGCTTAAGCTATTTCTGACTTCGTCTTTAATGC
TTGAAAGGACTTCATATTACAGGTTACGGGTTTTCTCTTGATATTTTCATGCAGAAAGCAAAG
TGTTTTGATATGCGTAAAAAAAAAAAGCTT

F12

AAGCTTTTTTTTTTTTATAAGGTTCATCTACTAACGATTATGCGCACGCACTATAAGGTAGATG
GTAACATATTTTACATTAATATCATTATCCCATGATATCGTGGGGAGAATGTACCACAGGATT
TGGAGTACATTGTTTACATTATTTATTTCCATAAGGTTTAATGTCATGCCACCACTGCTTGCCA
AGTGGCATACCTACCGGTTCACTATGCTGGCGACTGTGACTGCCCTTTATTGTCCTGACC
AAAGCTT

F13 (CA844438)

AAGCTTTGGTCAGGACAATAAAGGGCAGTCACAGTCGCCAGCATAGTGAACCGGTAGGTAT
GCCACTTGGCAAGCAGTGGTGGCATGACATTAAACCTTATGGAAATAAATAATGTAAACAAT
GTACTCCAAATCCTGTGGTACATTCTCCCCACGATATCATGGGATAATGATATTAATGTAAAA
TATGTTACCATCTACCTTATAGTGCGTGCGCATAATCGTTAGTAGATGAACCTTATAAAAAAA
AAAAGCTT

F14 (CA844403)

AAGCTTTTTTTTTTTTATGATTCGGGAACAATCCATGACCCTCGGTTGTCTGAAGGGCAGGGG
GAGGAGAGGCGGTTGGAGGAGATCAGGGACGACGACTTTAAGCACCGATCGCGTCTGACT
CAAGAAGAGCTCGCCTTGCTTGTCGAGCGATATTGGCAGGATGTGTGGCAGTATGCGTATT
TTCTGACCAAAGCTT

F15 (CA844404)

AAGCTTTTTTTTTTTTATACAACTTCATCTATTTCTTDGAGTTCATCTCTTATTCTAGATATCCTT
AGAAAGTATGATGAAACAATATCATCTTTAATCATCTTTATTTTCCTTAGTTCATTCTTGAGTCT
ACCTATCTCACCAAAGCTT

F16 (CA844404)

AAGCTTTGGTCAGGGGATCAAAGGTTTCAGTTGCAGGAGATATAAAGGAGGAGGGTAGTTAT
GATTTTGTGATACTTGGGGTTTTTGTCGATGTAGTGTAGGGTGGAAAAGTAGAGATCAAAAT
GTCATGGAAAGAATGAATTCATTTATCAAAAGACAAGAGACATAAACATGAAGTTCCTAAAAA
AAAAAAGCTT

F17 (CA844435)

AAGCTTTGGTCAGGAGAAGAAGAATATGTAGTAGCAACATCAACAACATGTCAAGTAGTGTA
GTTAAGAAGAATACTTGATGAACTCAAGCATAAAACTTAAAAAAAAACTTTCTATAAAATCCTT
TCCGAAATTTTGTTTACTTATTAAAAAATAAATTATAAAATAAATCCTTTTCAACATATAAA CCA
ATTATGGGAATATTTTCACCATATAAACTAGCAACGTAAAAAAAAAAAGCTT

F18 (CA844436)

AGCTTTTTTTTTTTTTACCATTCGCTTTTGTCTCTCTTTCTGTCAGCCTTTGCCCCTCCCGCCC
CCCCCCAATGTCTTGGCCCCTTTCCCGGGTCCCCCTTCCCCCCTTAGCCTTGCCCCCATGG
CCTTTTCCCTGCCCCACGCTT

F19 (CA844437)

AAGCTTTTTTCATTTTGAAAAAGGATATTATTTGTCGAGCTGCATGCTTTGTAGCATATATGTA
TCTGGTCACATTCGATTGGATAGTACTTGGAGTTTGCATTTAATTTATATATGCAGATAGAAC
ATCCAACGTTCTTACGTNCTCTCGTCTACCACACACTTGTTT

F20 (CA844406)

AAGCTTTGGTCAGATAAAGCGTGTTATCTTGAAAAAAAAAAAAACCTTGAATCATCAAGCATGC
ACGCATTTTGACATTTTTTTCAATCAAATGTTAGAAAATAATCTGGCTATTTTAGTCCTTTTGT
GGTAGTAAATAATATTTTAATTGGCTTGTAAAAAAAAAAAGCTT

F21 (CA844407)

AAGCTTTTTTTTTTTACAAGCCAATTAAAATTTTATTTACTACCACAAAAGGCCTAAAATAGCC
AGATTATTTTCTAACATTTGATTGAAAAAAATGTCAAAATGCGTGCATGCTTGATGATTCAAGT
TTTTTTTTCAAGATAACACGCTTTTTCTGACCCAAGCTT

F22 (CA844408)

AAGCTTTGGTCAGCATTGCCTTGCCTTTCTCTATATAAAGTATAAATATAGTGGAATAAACTC
CGCCAACGCGTAGGCGATCAAGTAGGCAGAATGACTTTTGTCTTTGGAAATTGAAATAATAT
TAATGATGTTGTAAAAAAAAAA AGCTT

F23 (CA844409)

AAGCTTTTTTTTTTAGTATCTACTCAATTCTGATTACTTGAATAAAGAAAGAGAAGCATGCCGA
AGTTACAATAACAGAATAGTAAAATAATAATTAAATTTCCCTTTTACTCCAAAATGCCCGAAAT
TAAAGCCACTT

F24 (CA844410)

AAGCTTTGGTCAGCACAGAGTCTACCGTCTAGCTAATAAATAAATAAATAAAATGAGAACTGC
GATAAGTAGTGAGGTTCGCTAACTGGATTGCGCCCCCCTCCCTGCAAAGTAATATAGAGTAG
ATTAATTATTTATATCAGTAACGTCACTCATGTTTTCCTACAATAAGGAAGAAATAAAGCGGAT
CACCTTTGGCATGGTAGCCAAATATTTTTCCCTAAAAAAAA AAAGCTT

F25 (CA844411)

AAGCTTTTTTTTTTTTACAGAAATGTTTGCTTTATCATTTTATTTTGTAAGATAAATACGTTGAAA
TGTCCTCTTTCCCTTGTAACTTGTACGGTGCATATTTCTGAGTTCATTCATGCACAACTTCTAA
CCTATACAGAAATTACAAGCATTCTTATGACTTCTGCAACAGCACTGGATTCAAGCCAGATTC
CTCAACCTCCATGCACAACTTCTAACCTATACAGAATTACAAGCATTCTCATGCTTCTGCAAG
CACTARATTCAAGCCAGATTACCTCAACCTCTTTGCCTCTTTGGAGGCAAAAATCCACCTCTG
ACCAA AGCTT

F26 (CA844412)

AAGCTTTTTTTTTTTTACAATAACAGAATAGTAAAATAATAATTAAATTTCCCTTTTACTCCAAAA
TGCCCGAAATTAAAGCCACTTGGCTAACATACCCCTAAACACAAACAAAAATGTAATGTCCT
GACCAAAGCTT

F27 (CA844413)

AAGCTTTGGTCAGGACATTACATTTTTGTTTGTGTTTAGGGGTATGTTAGCCAAGTGGCTTTT
AATTTCGGGCATTTTGGAGTAAAAGGGAAATTTAATTATTATTTTACTATTCTGTTATTGTAAC
TTCGGCATGCTTCTCTTTCTTTATTCAAGTAATCAGAATTGAGTAGATACTAAACCCAGTTTTT
TTCTAAAAAAAAAAAGCTT

F28 (CA844414)

AAGCTTTTTTTTTTTTAGTACAATATATAGTTTAACCCTTCGGTAACCCCATTTCCAATCCCCGC
ACCTACCTACATTTTTCTTTGGTTAATGAATGTTTCCCCAATTTTTTCTTTGCTTGTTTTAGGGT
AAAGGCTTCCCGTAAAATCGCTACCCATGTCATGCTTTCAAAGCCCCTTCCCAATCTTATCTT
TCCTCATGTTCCCTTTCCTTGTAAAGCAATGCATGCTAACTCCCCGGTTCCTGCCCCAAGCTT
C

F29

CACGGCAACA CCGGTAATCT TGCAGTTCCC GTTGATAGGA GTCATAGGCC ATGAAGGATT
CAGCC

F30

AAGCTTTG GTCAGGGAGG CCACATCTTT ACTCTAAAAA AAAAAAGCTT

F31

AGCTTTG GTCAGGGAGG CCACATCTTT AAAAAAAAAA AGCTT

F32 (CA844415)

AGGAAGCTTTTTTTTTTTTACCACATCATTAATATTATTTCAATTCCAAAGACAAAAGTCATTCTG
CCCACTTGATCGCCTACGGGTTGGCGGAGTTTATTCCACTATATTTATACTTTATATAGAGAA
GGCAAGGCAATGCTGACCAAAGCTT

F33 (CA844416)

AAGCTTTTTTTTTTTTAGAAAGACAAAATTCATAAAAATTCATTAGAGACCTTTATTAGATCATTT
ATTTGAACTTAATGGCACAACCGAACATTTAGTCCACCTTAGCCATGTTACTGACCAAAAGAT
TACTTTCAGGTACTTCTGACCGCCATCTATGACAAAAAAATTAAATGTTGCTTCGTTCGCGTC
AGTCAGTTTGGCTACAA AAGGCTTCCATTCACTGGCTCGCGTCCAG TAT

F34 (CA844417)

AAGCCTTTGTTGTAGCCAAACTGACTGACGCGAACGAAGCAACATTCAAGAAACTGGTCAT
AGATGGCGGTCAGAAGTACCTGAAAGG

F35 (CA844418)

AGGCTTTTTTTTTTTTAGCAAACCACGGTTACATTTTTGAAGCCAAGGAAGTGAAGAGAACTAG
CATGCTGACCAAAAGTTATCCTAATCGCTTAATGGCCTATGTAACTCCTATCAACGGAAAAAA
AAGGTTGTCGGTGTGTTGGCGTGGTAAGCGGGGGTTAAATTCGTTTGTTCAATTCTTCCCGC
CGCCAGGGCCTCGTCCCTTCC

F36 (CA844427)

AAGCTTTTTTTTTTTTAGGTGGGAAAGCATGAAGCCAAATCACTATTCAACACATTACATTATTC
ACACGGTAGTACAATATATAGTATAAACATTCGGTAAACACATCTACAATCCA CGGAACTAAC
TACATGTTTCTTAGGATAACGGAATATATCCCAAAGTTATTTATATTGCTAGTTTAAGACGTAA
AGACAAAAAAATAAATCGCTAACAATGTCATGCTTTCAAGCCCCTTCACAATCTTATCTAACAT
GTAACATAT CATTGTAAAGCAATGCATGCTAACTCCACGGTTCCTGACCAAAGCTT

F37 (CA844428)

AAGCTTTGGTCAAGAACATCCATGAAGAGACATCCCCATNCTTGTCAATGGGCATAGGTTGA
AAATCTATAGGAAGCCCTTATACAAGCAAGAATTCATTGAAAATCTGACTAAAACATTGTTGG
TAGTGGANCACCCCTTTGCCTCCACTCCACCCACACCCTAAAAAAAAA AAGCTTCC

F38 (CA844419)

AAGCTTTCTTTTTTTTTTTTACACCCAACAATCCCTATTATCACTTCACCTTTACCAAACCTTCTC
TCCTCTCCCCCCCGCCCACCACGCGACTCTCCCCTCTCCACACGCACTACCTCTCTCCTCTC
GCCCCCCCCACCTCTGACGTTCTGCCCCCCTGTGTGCTGTCTGTGTGTAGAATCTGGCTTG
GTTTTGGGTTCGCGCGCGCGCAGCTGCCAGCCATTGTCGCTGATATCCCACACCCAACG
GCTCTTCCCC TTTT

F39 (CA844420)

AAGTTTTTTTTTTTTATTCAAGGAGAGGGAATTTAGCATGCTTTATCTCGGGATACAAAAAAGA
GATTTATGAATTTCATATAAGGTAATGCTCTCTTTACCTCGAATTTAGGTCTCTCTCGTAGGTG
CACGGTGGTTGTTCACTCTACACACAAAGCCTTGCCTTTCTATGGGCTACTTTCTCTGCACC
CCCTGGTATTGCCCCTTTTCTCTTGAGATTGTTGTGTCTTGTGTGTGCTGGGGCAATTAGTTT
GGTACACACACCAAAGGGTCTTCCCT

F40 (CA844421)

AAGCTTTTTTTTTTTTACCGTCGAGAGCCTCAAACTAAAACTGTATTTCAGTTCACTGTTACCTG
GCCTATGCCGAGGACCTTAATACTTATTACAGAGGATTGCATGTCTGGTGCAAAGGGAATCA
ATCAAATATAAGCTAATTATAGCATTGAATCTGAGAACCTCAGAGAAACGTCCACTGCTGACC
AAAGCTT

Appendix 2. Results of the search for sequence similarity between the unknown fragments and known genes reported in the SIB. A Simplified map follows each figure showing segments of similarity.

a.

```
>emb|BM367151|BM367151 [Pinus taeda]NXLV_045_C12_F NXLV (Nsf Xylem
          Late wood Vertical) Pinus taeda cDNA clone NXLV_045_C12
          5', mRNA sequence.
         Length = 314

 Score =  297 bits (150), Expect = 4e-79
 Identities = 159/162 (98%)
 Strand = Plus / Minus


   F4: 13  agcttaagctgcagagacacggcataacaattaacaggccattaacggtacaagccatac 72
           |||||||||||||||||||| |||||| ||||||||||||||||||||||||||||||||
 Sbjct: 216 agcttaagctgcagagacacagcataataattaacaggccattaacggtacaagccatac 157


   F4: 73  tgaaagtcccaagactacctaaactgaagtctttatttatttacgagtgcaggtagagga 132
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 Sbjct: 156 tgaaagtcccaagactacctaaactgaagtctttatttatttacgagtgcaggtagagga 97


   F4: 133 gcctccatggccctgcacaaccatccattgggtctgaccaaa 174
           ||||| ||||||||||||||||||||||||||||||||||||
 Sbjct: 96  gcctctatggccctgcacaaccatccattgggtctgaccaaa 55
```

b.

```
emb|BM367151|BM367151 [Pinus taeda]NXLV_045_C12_F NXLV (Nsf Xylem
          Late wood Vertical) Pinus taeda cDNA clone NXLV_045_C12
          5', mRNA sequence.
         Length = 314

 Score =  331 bits (167), Expect = 2e-89
 Identities = 183/187 (97%), Gaps = 1/187 (0%)
 Strand = Plus / Plus


   3D: 1   ttggtcagacccaatggatggttgtgcagggccatggaggctcctctacctgcactcttt 60
           |||||||||||||||||||||||||||||||||||| |||||||||||||||||||||| |
 Sbjct: 56  ttggtcagacccaatggatggttgtgcagggccatagaggctcctctacctgcactcgt- 114


   3D: 61  aaataaataaagacttcagtttaggtagtcttgggactttcagtatggcttgtaccgtta 120
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 Sbjct: 115 aaataaataaagacttcagtttaggtagtcttgggactttcagtatggcttgtaccgtta 174


   3D: 121 atggcctgttaattattatgctgtgtctctgcagcttaagctatttctgacttcgtcttt 180
           |||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
 Sbjct: 175 atggcctgttaattattatgctgtgtctctgcagcttaagctatttctaacttcgtcttt 234


   3D: 181 aaatgct 187
           |||||||
 Sbjct: 235 aaatgct 241
```

c.

```
>emb|BM367151|BM367151 [Pinus taeda]NXLV_045_C12_F NXLV (Nsf Xylem
          Late wood Vertical) Pinus taeda cDNA clone NXLV_045_C12
          5', mRNA sequence.
         Length = 314

 Score =  367 bits (185), Expect = e-100
 Identities = 237/253 (93%), Gaps = 3/253 (1%)
 Strand = Plus / Plus


   F10: 5   tttggtcagacccaatagatggttgtgcagggccatagaggctactctaccttcactcgt 64
            |||||||||||||||| |||||||||||||||||||||||| |||||||| |||||||
 Sbjct: 55  tttggtcagacccaatggatggttgtgcagggccatagaggctcctctacctgcactcgt 114


   F10: 65  aaataaatcaagacttcggtttaggtagtcttgggactttcagtatggcttgtaccgtta 124
            |||||||| |||||||| ||||||||||||||||||||||||||||||||||||||||
 Sbjct: 115 aaataaataaagacttcagtttaggtagtcttgggactttcagtatggcttgtaccgtta 174


   F10: 125 atggcatgttaattattatgctgtgtctctgcagcttaagctatttctgacttcgtcttt 184
            ||||| ||||||||||||||||||||||||||||||||||||||| ||||||||||||
 Sbjct: 175 atggcctgttaattattatgctgtgtctctgcagcttaagctatttctaacttcgtcttt 234


   F10: 185 -aatgcttgaaaggacttc--atattacaggttacgggttttctcttgatattttcatgc 241
             |||||||||||||||||  |||||||  |   |||||||||||||||||||||||  ||
 Sbjct: 235 aaatgcttgaaaggacttcatatattaccgnnnacgggttttctcttgatattttcacgc 294


   F10: 242 agaaagcaaagtg 254
            | ||||||||||
 Sbjct: 295 acaaagcaaagtg 307
```

Figure 6-1. Nucleotide sequence alignments between *P. taeda* cDNA clone NXLV_045_C12 5' mRNA and fragment (a) F4, (b) 3D, (c) F10.

Pinus taeda cDNA clone NXLV_045_C12 5', mRNA sequence (Nsf Xylem Late Wood Vertical) Length = 314

```
>emb|AW754974|AW754974 [Pinus taeda]PC09A05 Pine TriplEx pollen cone
          library Pinus taeda cDNA clone PC09A05, mRNA sequence.
         Length = 380

 Score =  210 bits (106), Expect = 3e-53
 Identities = 109/110 (99%)
 Strand = Plus / Plus


   5D: 30  cagaatctaatccgtcggttaccttgtccatgaaagggagtatatatctttaaacgagcg 89
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 Sbjct: 1  cagaatctaatccgtcggttaccttgtccatgaaagggagtatatatctttaaacgagcg 60


   5D: 90  acattaaaaacttacaatgctctttgttgaaatagaagggttttcttact 139
           ||||||||||||||||||||| ||||||||||||||||||||||||||||
 Sbjct: 61  acattaaaaacttacaatgctgtttgttgaaatagaagggttttcttact 110
```

Figure 6-2. Nucleotide sequence alignments between *P. taeda* cDNA clone PC09A05 mRNA sequence and fragment 5D.

PCO1A07 Pine triplEx pollen cone library *Pinus taeda* cDNA clone PC09A05, mRNA sequence. Length = 380

1_____380

1_____110 P. *taeda*
30_____139 5D

a.

```
>emb|AW010057|AW010057 [Pinus taeda]PC01A07 Pine TriplEx pollen cone
          library Pinus taeda cDNA clone PC01A07, mRNA sequence.
         Length = 291

 Score = 60.2 bits (30), Expect = 6e-08
 Identities = 37/38 (97%), Gaps = 1/38 (2%)
 Strand = Plus / Minus


   5E: 125 tgaaatagaaccaaatttatatcattctgctctttccc 162
           |||||||||||||||||| ||||||||||||||||||||
 Sbjct: 178 tgaaatagaaccaaatt-atatcattctgctctttccc 142
```

b.

```
>emb|AW010057|AW010057 [Pinus taeda]PC01A07 Pine TriplEx pollen cone
          library Pinus taeda cDNA clone PC01A07, mRNA sequence.
         Length = 291

 Score = 69.9 bits (35), Expect = 1e-10
 Identities = 38/39 (97%)
 Strand = Plus / Plus


   F2: 14  gggaacgagcagaatgatataatttggttctatttcatc 52
           |||||| ||||||||||||||||||||||||||||||||
 Sbjct: 142 gggaaagagcagaatgatataatttggttctatttcatc 180
```

Figure 6-3. Nucleotide sequence alignments between *P. taeda* cDNA clone PC01A07 mRNA sequence and fragment (a) 5E, (b) F2.

PC01A07 Pine tripIEx pollen cone library *Pinus taeda* cDNA clone PC01A07, mRNA sequence. Length = 291

1 _____ 291

142 _____ 178 P taeda
162 _____ 125 5E
142 _____ 180 P taeda
14 _____ 52 F2

a.

```
>emb|AA739899|AA739899 [Pinus taeda]664 PtIFG2 Pinus taeda cDNA
          clone 9079M 3', mRNA sequence.
         Length = 351

 Score = 210 bits (106), Expect = 2e-53
 Identities = 109/110 (99%)
 Strand = Plus / Plus


   4B: 6   ttggtcagctaataaaaacatccatatcattatatggaggaggtggtggagttgattcgg 65
           |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 Sbjct: 242 ttggtcagctaataaaaacatccatatcattatatggaggaggtggtggagttgattcgg 301


   4B: 66  agcatagaagataccatataacattataaaatatgtggcttcatcttatt 115
           |||||||||||||||||||| ||||||||||||||||||||||||||||
 Sbjct: 302 agcatagaagataccatataatattataaaatatgtggcttcatcttatt 351
```

b.

```
>emb|AA739899|AA739899 [Pinus taeda]664 PtIFG2 Pinus taeda cDNA
          clone 9079M 3', mRNA sequence.
         Length = 351

 Score =  210 bits (106), Expect = 2e-53
 Identities = 109/110 (99%)
 Strand = Plus / Plus


  5A: 6   ttggtcagctaataaaaacatccatatcattatatggaggaggtggtggagttgattcgg 65
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 242 ttggtcagctaataaaaacatccatatcattatatggaggaggtggtggagttgattcgg 301


  5A: 66  agcatagaagataccatataacattataaaatatgtggcttcatcttatt 115
          ||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct: 302 agcatagaagataccatataatattataaaatatgtggcttcatcttatt 351
```

Figure 6-4. Nucleotide sequence alignments between *P. taeda* cDNA clone 9079M 3' mRNA sequence and fragment (a) 4B, (b) 5A

664 OtIFG2 *Pinus taeda* cDNA clone 9079M 3' mRNA sequence. Length = 351

1			351
P taeda	242		351
4B	**6**		**115**
5A	**6**		**115**

```
>emb|BI740099|BI740099 [Cucumis sativus]EST0144 cucumber DD-RTPCR
          cDNA fragments Cucumis sativus cDNA clone CsSE25-1, mRNA
          sequence.
         Length = 109

 Score =  107 bits (54), Expect = 1e-22
 Identities = 57/58 (98%)
 Strand = Plus / Minus


  F29: 6   caacaccggtaatcttgcagttcccgttgataggagtcataggccatgaaggattcag 63
           |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 106 caacaccgataatcttgcagttcccgttgataggagtcataggccatgaaggattcag 49
```

Figure 6-5. Nucleotide sequence alignment between *P. taeda* cDNA clone CsSE25-1 mRNA sequence and fragment F29.

EST0144 cucumber DD-RTPCR cDNA fragments *Cucumis sativus* cDNA clone CsSE25-1, mRNA sequence. Length = 109

1			109
	P. taeda	49	106
	F29	**63**	**6**

a.

```
emb|BI740085|BI740085 [Cucumis sativus]EST0130 cucumber DD-RTPCR
          cDNA fragments Cucumis sativus cDNA clone CsSE10-3, mRNA
          sequence.
         Length = 162

 Score = 58.0 bits (29), Expect = 4e-07
 Identities = 29/29 (100%)
 Strand = Plus / Minus


  F33: 131 ctttcaggtacttctgaccgccatctatg 159
           |||||||||||||||||||||||||||||
 Sbjct: 31  ctttcaggtacttctgaccgccatctatg 3
```

b.

```
>emb|BI740085|BI740085 [Cucumis sativus]EST0130 cucumber DD-RTPCR
          cDNA fragments Cucumis sativus cDNA clone CsSE10-3,
          mRNA sequence.
          Length = 162

 Score = 60.0 bits (30), Expect = 3e-08
 Identities = 30/30 (100%)
 Strand = Plus / Plus


  F34: 59 catagatggcggtcagaagtacctgaaagg 88
          ||||||||||||||||||||||||||||||
 Sbjct: 3  catagatggcggtcagaagtacctgaaagg 32
```

Figure 6-6. Nucleotide sequence alignments between *P. taeda* cDNA clone CsSE10-3 mRNA sequence and fragment (a) F33, (b) F34

EST0130 cucumber DD-RTPCR cDNA fragments *Cucumis sativus* cDNA clone CsSE10-3, mRNA sequence. Length = 162.

1 _____ 162

3	31	*C. sativus*
159	131	F 33
3	32	*C. sativus*
59	88	F34

```
>emb|AW758937|AW758937 [Pinus taeda]NXNV_095_E08_F Nsf Xylem Normal
          wood Vertical Pinus taeda cDNA clone NXNV_095_E08 5',
          mRNA sequence.
         Length = 253

 Score = 73.8 bits (37), Expect = 8e-12
 Identities = 54/62 (87%)
 Strand = Plus / Plus


   2E: 7   tggtcaggtatgagtcctgtcctggtgaactgataaatgttttgaatttctgaatcagtt 66
           |||| ||||||||||||||||||  ||||||||||||||  | ||||||||||||  |||
 Sbjct: 192 tggttaggtatgagtcctgtcctnntgaactgataaatgnntngaatttctgaatnngtt 251


   2E: 67  tt 68
           ||
 Sbjct: 252 tt 253
```

Figure 6-7. Nucleotide sequence alignments between *P. taeda* cDNA clone NXNV_095_E08 5' mRNA sequence and fragment 2E.

NXNV_095_E08_F Nsf Xylem Normal wood Vertical *Pinus taeda* cDNA clone NXNV_095_E08 5', mRNA sequence. Length = 253

192_____253

P. taeda 192_____253

2E **7**_____**68**

```
>emb|BF610506|BF610506 (Pinus taeda)NXSI_059_D08_F NXSI (Nsf Xylem
          Side wood Inclined) Pinus taeda cDNA clone NXSI_059_D08
          5', mRNA sequence.
         Length = 303

 Score =  174 bits (88), Expect = 1e-42
 Identities = 100/104 (96%)
 Strand = Plus / Minus


   4A: 16  actgaaagtcccaagactacctaaaccgaagtcttgatttatttacgagtgaaggtagag 75
           ||||||||||||||||||||||||| ||||||| |||||||||||| | ||||||||
 Sbjct: 232 actgaaagtcccaagactacctaaactgaagtctttatttatttacgagggcaggtagag 173


   4A: 76  tagcctctatggccctgcacaaccatctattgggtctgaccaaa 119
           |||||||||||||||||||||||||||||||||||||||||||
 Sbjct: 172 tagcctctatggccctgcacaaccatctattgggtctgaccaaa 129
```

Figure 6-8. Nucleotide sequence alignment between *P. taeda* cDNA clone NXSI_059_D08 5' mRNA sequence and fragment 4A.

***Pinus taeda* cDNA clone NXSI_059_D08 5', mRNA sequence. Length = 303**

1_____303

P. taeda 129_____232

4A **119**_____**16**

a.

```
>emb|BM158310|BM158310 [Pinus taeda]NXLV_032_D03_F NXLV (Nsf Xylem
          Late wood Vertical) Pinus taeda cDNA clone NXLV_032_D03
          5', mRNA sequence.
         Length = 457

 Score = 95.6 bits (48), Expect = 1e-18
 Identities = 65/73 (89%)
 Strand = Plus / Plus


  F20: 44  ccttgaatcatcaagcatgcacgcattttgacannnnnnncaatcaaatgttagaaaata 103
           ||||||||||||||||||||||||||||||||||        || ||||||||||||||||
Sbjct: 385 ccttgaatcatcaagcatgcacgcattttgacatttttttcagtcaaatgttagaaaata 444


  F20: 104 atctggctatttt 116
           |||||||||||||
Sbjct: 445 atctggctatttt 457
```

b.

```
>emb|BM158310|BM158310 [Pinus taeda]NXLV_032_D03_F NXLV (Nsf Xylem
          Late wood Vertical) Pinus taeda cDNA clone NXLV_032_D03
          5', mRNA sequence.
         Length = 457

 Score = 95.6 bits (48), Expect = 1e-18
 Identities = 65/73 (89%)
 Strand = Plus / Minus


  F21: 56  aaaatagccagattattttctaacatttgattgnnnnnnntgtcaaaatgcgtgcatgct 115
           |||||||||||||||||||||||||||||||| ||       ||||||||||||||||||||
Sbjct: 457 aaaatagccagattattttctaacatttgactgaaaaaaatgtcaaaatgcgtgcatgct 398


  F21: 116 tgatgattcaagg 128
           |||||||||||||
Sbjct: 397 tgatgattcaagg 385
```

Figure 6-9 Nucleotide sequence alignments between *P. taeda* cDNA clone NXLV_032_D03 5' mRNA sequence and fragment a) F20 b) F21.

***Pinus taeda* cDNA clone NXLV_032_D03 5', mRNA sequence. Length = 457**

1_____457

P.taeda	385_____	457
F20	**44_____**	**116**
P taeda	385_____	457
F21	**128_____**	**56**

a.

```
>emb|AW225849|AW225849 [Pinus taeda]ST72D12 Pine TriplEx shoot tip
           library Pinus taeda cDNA clone ST72D12, mRNA sequence.
          Length = 467

 Score =  168 bits (85), Expect = 2e-40
 Identities = 98/102 (96%), Gaps = 1/102 (0%)
 Strand = Plus / Minus


  F36: 204 aaatcgctaacaatgtcatgctttcaa-gccccttcacaatcttatctaactcatgtaac 262
           ||||||||   |||||||||||||||| |||||||||||||||||||||||||||||||
 Sbjct: 415 aaatcgctnnnaatgtcatgctttcaaagccccttcacaatcttatctaactcatgtaac 356


  F36: 263 atatcattgtaaagcaatgcatgctaactccacggttcctga 304
           |||||||||||||||||||||||||||||||||||||||||
 Sbjct: 355 atatcattgtaaagcaatgcatgctaactccacggttcctga 314
```

b.

```
>emb|AW225849|AW225849 [Pinus taeda]ST72D12 Pine TriplEx shoot tip
           library Pinus taeda cDNA clone ST72D12, mRNA sequence.
          Length = 467

 Score = 95.6 bits (48), Expect = 2e-18
 Identities = 91/105 (86%), Gaps = 1/105 (0%)
 Strand = Plus / Minus


  F28: 142 gtaaaatcgctacccatgtcatgctttcaaagccccttcccaatcttatctttcctcatg 201
           ||||||||||||    |||||||||||||||||||||||| |||||||||| |  ||||||
 Sbjct: 418 gtaaaatcgctnnnaatgtcatgctttcaaagccccttcacaatcttatc-taactcatg 360


  F28: 202 ttccctttccttgtaaagcaatgcatgctaactccccggttcctg 246
           |  | | || |||||||||||||||||||||||| |||||||||
 Sbjct: 359 taacatatcattgtaaagcaatgcatgctaactccacggttcctg 315
```

Figure 6-10. Nucleotide sequence alignments between *P. taeda* cDNA clone ST72D12 mRNA sequence and fragment a) F36 b) F28.

ST72D12 Pin TriplEx shoot tip library *Pinus taeda* cDNA clone ST72D12, mRNA sequence. Length = 467

1_____467

P.taeda	314	415
F36	**304**	**204**
P.taeda	315	418
F28	**246**	**142**

a.

```
>emb|BM158362|BM158362 [Pinus taeda]NXLV_033_C08_F NXLV (Nsf Xylem
          Late wood Vertical) Pinus taeda cDNA clone NXLV_033_C08
          5', mRNA sequence.
         Length = 650

 Score =  242 bits (122), Expect = 7e-63
 Identities = 124/125 (99%)
 Strand = Plus / Minus


  F23: 15  agtatctactcaattctgattacttgaataaagaaagagaagcatgccgaagttacaata 74
           |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
 Sbjct: 470 agtatctactcaattctgattacttgaataaagnaagagaagcatgccgaagttacaata 411


  F23: 75  acagaatagtaaaataataattaaatttccctttactccaaaatgcccgaaattaaagc 134
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 Sbjct: 410 acagaatagtaaaataataattaaatttccctttactccaaaatgcccgaaattaaagc 351


  F23: 135 cactt 139
           |||||
 Sbjct: 350 cactt 346
```

b.

```
>emb|BM158362|BM158362 [Pinus taeda]NXLV_033_C08_F NXLV (Nsf Xylem
          Late wood Vertical) Pinus taeda cDNA clone NXLV_033_C08
          5'  mRNA sequence.
         Length = 650

 Score =  208 bits (105), Expect = 9e-53
 Identities = 105/105 (100%)
 Strand = Plus / Minus


   5B: 16  agtaaaataataattaaatttccctttactccaaaatgcccgaaattaaagccacttgg 75
           |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 Sbjct: 403 agtaaaataataattaaatttccctttactccaaaatgcccgaaattaaagccacttgg 344


   5B: 76  ctaacatacccctaaacacaaacaaaaatgtaatgtcctgaccaa 120
           ||||||||||||||||||||||||||||||||||||||||||||
 Sbjct: 343 ctaacatacccctaaacacaaacaaaaatgtaatgtcctgaccaa 299
```

c.

```
>emb|BM158362|BM158362 [Pinus taeda]NXLV_033_C08_F NXLV (Nsf Xylem
          Late wood Vertical) Pinus taeda cDNA clone NXLV_033_C08
          5', mRNA sequence.
        Length = 650

 Score =  335 bits (169), Expect = 9e-91
 Identities = 171/172 (99%)
 Strand = Plus / Plus


   5F: 6   ttggtcaggacattacatttttgtttgtgtttaggggtatgttagccaagtggctttaat 65
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 Sbjct: 299 ttggtcaggacattacatttttgtttgtgtttaggggtatgttagccaagtggctttaat 358


   5F: 66  ttcgggcattttggagtaaaagggaaatttaattattattttactattctgttattgtaa 125
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 Sbjct: 359 ttcgggcattttggagtaaaagggaaatttaattattattttactattctgttattgtaa 418


   5F: 126 cttcggcatgcttctctttctttattcaagtaatcagaattgagtagatact 177
           |||||||||||||||||| ||||||||||||||||||||||||||||||
 Sbjct: 419 cttcggcatgcttctcttnctttattcaagtaatcagaattgagtagatact 470
```

d

```
>emb|BM158362|BM158362 [Pinus taeda]NXLV_033_C08_F NXLV (Nsf Xylem
          Late wood Vertical) Pinus taeda cDNA clone NXLV_033_C08
          5', mRNA sequence.
        Length = 650

 Score =  327 bits (165), Expect = 2e-88
 Identities = 170/172 (98%)
 Strand = Plus / Minus


   6C: 16  agtatctactcaattctgattacttgaataaagaaagagaagcatgccgaagttacaata 75
           |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
 Sbjct: 470 agtatctactcaattctgattacttgaataaagnaagagaagcatgccgaagttacaata 411


   6C: 76  acagaatagtaaaataataattaaatttccctttactccaaaatccccgaaattaaagc 135
           |||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
 Sbjct: 410 acagaatagtaaaataataattaaatttccctttactccaaaatgcccgaaattaaagc 351


   6C: 136 cacttggctaacataccccctaaacacaaacaaaaatgtaatgtcctgaccaa 187
           |||||||||||||||||||||||||||||||||||||||||||||||||||||
 Sbjct: 350 cacttggctaacataccccctaaacacaaacaaaaatgtaatgtcctgaccaa 299
```

e.

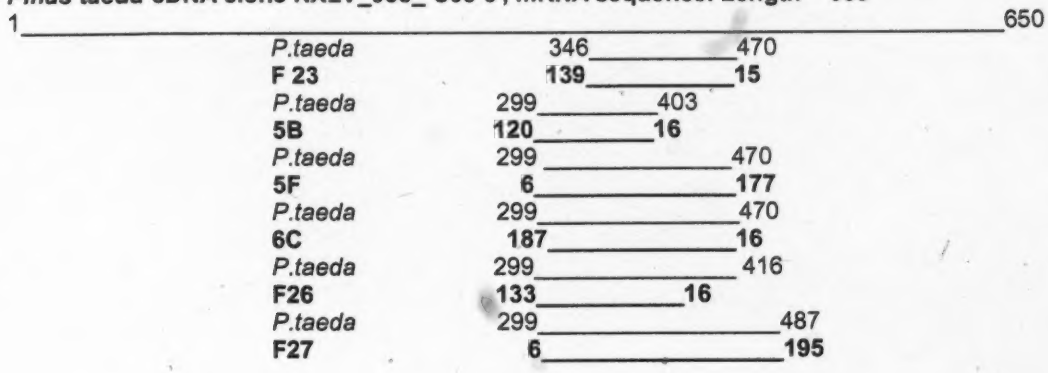
```
emb|BM158362|BM158362 [Pinus taeda]NXLV_033_C08_F NXLV (Nsf Xylem
          Late wood Vertical) Pinus taeda cDNA clone NXLV_033_C08
          5', mRNA sequence.
         Length = 650

 Score =  234 bits (118), Expect = 2e-60
 Identities = 118/118 (100%)
 Strand = Plus / Minus


  F26: 16  acaataacagaatagtaaaataataattaaatttccctttactccaaaatgcccgaaat 75
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 416 acaataacagaatagtaaaataataattaaatttccctttactccaaaatgcccgaaat 357


  F26: 76  taaagccacttggctaacataccctaaacacaaacaaaaatgtaatgtcctgaccaa 133
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 356 taaagccacttggctaacataccctaaacacaaacaaaaatgtaatgtcctgaccaa 299
```

f.

```
emb|BM158362|BM158362 [Pinus taeda]NXLV_033_C08_F NXLV (Nsf Xylem
          Late wood Vertical) Pinus taeda cDNA clone NXLV_033_C08
          5', mRNA sequence.
         Length = 650

 Score =  355 bits (179), Expect = 1e-96
 Identities = 188/190 (98%), Gaps = 1/190 (0%)
 Strand = Plus / Plus


  F27: 6   ttggtcaggacattacatttttgtttgtgtttaggggtatgttagccaagtggcttttaa 65
           |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct: 299 ttggtcaggacattacatttttgtttgtgtttaggggtatgttagccaagtggcttt-aa 357


  F27: 66  tttcgggcattttggagtaaaagggaaatttaattattatttactattctgttattgta 125
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 358 tttcgggcattttggagtaaaagggaaatttaattattatttactattctgttattgta 417


  F27: 126 acttcggcatgcttctctttctttattcaagtaatcagaattgagtagatactaaaccca 185
           |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct: 418 acttcggcatgcttctcttnctttattcaagtaatcagaattgagtagatactaaaccca 477


  F27: 186 gtttttttct 195
           ||||||||||
Sbjct: 478 gtttttttct 487
```

Figure 6-11. Nucleotide sequence alignments between *P. taeda* cDNA clone NXLV_033_C08 5' mRNA sequence and fragment a) F23 b) 5B c) 5F d) 6C e) F26 f) F27.

***Pinus taeda* cDNA clone NXLV_033_ C08 5', mRNA sequence. Length = 650**



Appendix 3. Analysis of the result of the RT-PCR confirming expression of fragments F3, F10, 1C, 5D, F24, F25, F27, F28, F36, F39

F3 from JP F111 T2

Target Tissue/Treatment	Relative Adjusted Volume		T-Test
T1 (0.5%NaCl)	**Control**	**Treated**	Pr>/t/=0.3046
JP4F-10	82.154	44.874	
JP4F-10	14.375	4.1969	
JP4EX-5	76.763	84.425	
JP6E-9	37.649	24.012	
T2 (0.7%NaCl)			
JP4F-12	38.326	16.295	
JP6E-5	39.668	10.018	

F10 from JP F111 T2

Target Tissue/Treatment	Relative Adjusted Volume		T-Test
T1 (0.5%NaCl)	**Control**	**Treated**	Pr>/t/=0.6818
JP1Da-7	3.36	0	
JP1Da-7	4.64	47.84	
JP2C-9	0.48	0	
JP2C-9	4	2.56	
JP3BA002a	12.96	0	
JP4F-10	43.04	0.16	
JP6E-9	10.56	11.04	
T2 (0.7%NaCl)			
JP4F-12	1.6	2.24	
JP6E-5	8.8	0	

1C from BS F5 T1

Target Tissue/Treatment	Relative Adjusted Volume		T-Test
T1 (0.5% NaCl)	**Control**	**Treated**	Pr>/t/=0.6703
JP1Da-7	485.67	517.89	
JP1Da-7	470.69	309.6	
JP2C-9	255.24	123.35	
JP2E-3	0	449.96	
JP3BA002a	496.38	375.8	
JP4F-10	361.22	482.69	
JP6E-9	412.29	300.42	
T2 (0.7% NaCl)			
JP6E-5	249.57	431.59	

5D from JP2D-10T3

Target Tissue/Treatment	Relative Adjusted Volume		T-Test
T1 (0.5%NaCl)	**Control**	**Treated**	Pr>/t/=0.1890
JP2C-9	63.318	115.14	
JP4F-10	9.28	43.172	
JP6E-9	1.4118	25.506	
T2 (0.7% NaCl)			
JP4F-12	14.024	47.219	

F24 from JP3Da-5C T3

Target Tissue/Treatment	Relative Adjusted Volume		T-Test
T1 (0.5% NaCl)	**Control**	**Treated**	Pr>/t/=0.0757
JP1Da-7	88.432	239.78	
JP2C-9	25.376	70.224	
JP2E-3	297.22	123.78	
JP2E-3	4.96	60.12	
JP2E-3	18.678	1167.3	
JP2E-3	10.029	645.9	
JP3BA002a	13.168	309.9	
JP6E-9	64.267	29.6	
T2 (0.7% NaCl)			
JP6E-5	0	99.296	
T3 (1.0% NaCl)			
JP3Da-5	12.107	89.6	
JP3Da-5	15.147	65.28	
JP3Da-5	17.12	98.827	
JP4G-1	95.872	9.328	

F27 from JP2Bb T3

Target Tissue/Treatment	Relative Adjusted Volume		T-Test
T1 (0.5% NaCl)	**Control**	**Treated**	Pr>/t/=0.7013
JP1Da-7	213.4	82.701	
JP1Da-7	68.875	65.421	
JP2C-9	18.151	4.9778	
JP2E-3	15.751	74.116	
T1 (0.5% NaCl)	**Control**	**Treated**	
JP3BA002a	20.809	0	
JP6E-9	38.844	32.213	
T2 (0.7% NaCl)			
JP4F-12	28.676	32.284	
JP6E-5	6.8978	36.16	

F25 from JP2Bb T3

Target Tissue/Treatment	Relative Adjusted Volume		T-Test
T1 (0.5% NaCl)	**Control**	**Treated**	Pr>/t/=0.0204
JP1Da-7	0	96.658	
JP1Da-7	130.63	85.849	
JP2C-9	2.9333	29.867	
JP2C-9	7.6267	8.32	
JP2C-9	7.5733	22.347	
JP2C-9	17.12	0	
JP2E-3	0	124.84	
JP2E-3	0	154.29	
JP2E-3	2.56	20.107	
JP2E-3	9.1733	164.48	
JP3BA002a	3.0933	170.13	
JP6E-9	21.7	27.04	
JP6E-9	95.147	69.013	
JP6E-9	61.28	64.107	
JP6E-9	74.027	42.293	
T2 (0.7% NaCl)			
JP6E-5	9.1733	29.76	
JP6E-5	0	1.6533	
JP6E-5	0	37.618	

F28 from JP2Bb T3

Target Tissue/Treatment	Relative Adjusted Volume		T-Test
T1 (0.5% NaCl)	**Control**	**Treated**	Pr>/t/=0.3039
JP1Da-7	0	3.52	
JP6E-5	0	1.12	

F39 from JP6D 3T3

Target Tissue/Treatment	Relative Adjusted Volume		T-Test
T1 (0.5% NaCl)	**Control**	**Treated**	Pr>/t/=0.8600
JP2E-3	28.049	38.634	
JP4F-12	82.843	86.855	

F36 from BS 2C-4T1

Target Tissue/Treatment	Relative Adjusted Volume		T-Test
T1 (0.5% NaCl)	**Control**	**Treated**	Pr>/t/=0.01517
JP1Da-7	81.829	224.5	
JP2C-9	219.7	318.51	
JP2E-3	14.583	222.72	
JP3BA002a	47.474	114.51	
JP6E-9	242.33	258.26	
T2 (0.7% NaCl)			
JP4F-12	246.35	152.91	
JP6E-5	30.629	107.82	
T3 (1.0% NaCl)			
JP4G-1	178.22	188.39	